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

David E. Metzger¹, Chengyan Liu², Amin Sam Ziaie², Ali Naji², and Kenneth S. Zaret¹*

¹Institute for Regenerative Medicine, Institute for Diabetes Obesity and Metabolism, Department of Cell and Developmental Biology and ²Department of Surgery
University of Pennsylvania Perelman School of Medicine, Smilow Center for Translational Research, 3400 Civic Center Blvd, Philadelphia, PA 19104

Running title: Grgs beta over alpha cell fates
Keywords: Grg3, TLE3, β-cell, α-cell, monohormonal, endocrine, glucagon, insulin

*Corresponding Author
Dr. Ken Zaret, zaret@upenn.edu, 215-573-5813
(ABSTRACT)

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 the endogenous repressive mechanisms used to maintain β cell identity. Here, 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 further 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 similar 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.
(INTRODUCTION)

Much attention has been directed to generating functional pancreatic β cells from other sources, such as from ES cells, iPS cells, or by the conversion of non-β cell types. Developmental biology experiments have outlined the multistep differentiation process towards 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 about how the pancreas develops monohormonal β cells.

Repressive mechanisms are often 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 have many specific roles during invertebrate and vertebrate development (5-7).

Of the Groucho family members expressed in the 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 specify the correct number of β cells and maintaining β cell identity by recruiting HDAC1 and Dnmt3a to the Arx gene (11; 12). Since the misexpression of Arx converts β cells to α cells (13), the Grg3-containing repressive complex which normally represses Arx expression in β cells may help prevent β to α conversion. However, it is not known if Grg3 is the essential Groucho protein acting during β cell induction and maturation. Furthermore, Grg3 may interact with other transcription factors that repress the α-cell fate. For example, Groucho proteins have previously been shown to bind Nkx6.1 in the context of neural tube development (14), and Nkx6.1 can repress the α cell fate (15).
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+ 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 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 via Grg1/TLE1 also occurs in human β cells. Our data show that Groucho/TLE corepressors may be useful sentinels of monohormonal β cell formation and as a tool along with other β cell transcription factors to efficiently convert α cells to functional β cells.
RESEARCH DESIGN AND METHODS

Immunofluorescence. Immunofluorescence (IF) 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-Grp3 (18), rabbit-α-Grp1 (18), and mouse-α-Nkx6.1 (BCBC) antibodies. To assess the specificity of αGrp3 and αGrp1 on human islet sections, antibodies were incubated with immunizing peptide (18) for 1 hr prior to application on sections. Cultured cells were fixed with 4% paraformaldehyde, permeabilized with 2% triton-X, blocked with 3% BSA and probed with rabbit-α-Grp3, mouse-α-Nkx6.1, goat-α-FoxA2 (Santa Cruz), mouse-α-Flag (Sigma), guinea pig-α-insulin, mouse-α-Pdx1 (BCBC), C-peptide (Cell Signaling) and Alexa Fluor-conjugated secondary antibodies (Invitrogen). Staining intensity of Grp1 on human islet sections was determined by analyzing random images of 15 α cells and 15 β cells using ImageJ software. Images were taken at the same exposure, and the same threshold was set for each image on ImageJ. Pixel area was then counted by ImageJ, and data is 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 FAC sorted GFP+ cells directly into RLT buffer and isolated RNA with RNeasy Mini Kit (Qiagen). To obtain RNA from neonatal α and β cells, YFP/GFP+ cells from Glucagon-Cre;R26R-YFP (21) and Pdx1-GFP (22) pancreata, respectively, were similarly sorted. All sorts were performed by the University of Pennsylvania FACS Facility.

Mouse genotyping. All animal studies were performed with IACUC approval. Mice were genotyped using the following primer sets: Grg3+/-(LacZ) 5'-
TACTGTCGTCGCCCATCAAA-3', 5'-ACTCCACGCAGCACCACCATC-3'; Ngn3-eGFP
5'-ATACTCCTGCTCCCGTG-3', 5'-TGGTTGCTGAGTGCAACTC-3', 5'-
GAACATTGCGCCGTTTACGT-3'; Glucagon-Cre 5'-
TTGAAATGCTTCTGTCCGTTTCGC-3', 5'-AACGAACCTGGTCCAGGACT-3'.

**Quantitative PCR for gene expression.** RT-qPCR was performed using the AgPath-ID One Step RT-PCR Kit (Ambion) using 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/Slut2 (Mm00446229_m1), Chga (Mm00514341_m1), Nkx6.1 (Mm00454962_m1), hPdx1 (Hs00236830_m1), hGapdh (Hs02758991_g1) and hTle3/Grg3 (Hs00183222_m1). Reactions were run on 7900HT Fast Real-Time PCR System (Applied Biosystems) and quantitated using the RQ Manager software (Applied Biosystems) using the ∆∆CT method. Expression was normalized to Gapdh, except for adult pancreas which was normalized with the pan-endocrine marker, Chga.

**Single cell sort and Fluidigm PCR.** Single GFP positive 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), 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 the above Taqman probes on the BioMark HD System (Fluidigm). The PCR results were evaluated by determining if gene expression was present in each cell.

**Lentiviral production, cell culture, knockdown and ectopic expression.** Grg3-specific (V2LMM_242222 and V2LHS_18355) and non-silencing 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 was passed through a 0.45 µM filter directly added to αTC1-6 and βTC6 cells. αTC1-6 and βTC6 cells were grown in DMEM (4.5 g/L glucose) with 10% FBS and PenStrep. For Grg3 knockdown in βTC6 cells, pGIPZ lentiviral media was directly added to cells. After 3 days the media was 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 cells were 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) and cultured for 3 days and sorted for negative, low and high GFP expressing cells into RLT buffer to isolate RNA.

**ChIP – qPCR.** ChIP was performed using 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) 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 $2^{(C_{\text{input}}-C_{\text{ChIP}})}$. Results are shown as ChIP/Input in relative units. PCR was performed on ChIP products for the Glucagon promoter, (5'-AAGCAGATGAGCAAAGTGAGTG-3' and 5'-AGGCTGTTTAGCCTTGAGATA-3'), Hnf1β promoter (5'-CTCTGGCAAGTCCCAATCCC-3' and 5'-CCATGATCTCCACCATTAGGC-3') and Hnf6 promoter (5'-TTTGGGCCATGACATAGTTTTC-3' and 5'-CTTGCTACCTCCTGGTCTTCC-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 by intraperitoneal injection. Blood glucose levels were measured with a Contour blood glucose meter (Bayer) at after fasting, after injections, and at timepoints 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 different species were used to probe for potential interacting proteins. Plus and Minus secondary antibodies detecting different primary antibodies from different species were then used. Plus and Minus antibodies in close 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 250ul of low-glucose (1 g/L) and high-glucose (4.5
g/L) serum-free DMEM for 1 hr at 37°. The media was then collected and insulin content was assessed using 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 the pancreatic endocrine cells in E15.5 and neonatal mice (8). We have now additionally analyzed α and β cells for Grg3 at E17.5 and adult. Immunofluorescence (IF) of the E17.5 pancreas shows that the vast majority of insulin+ β cells express Grg3 (Fig. 1A, red arrows, inset magnification in right panel) while glucagon+ α cells either lacked Grg3 or had low levels of nuclear Grg3 (Fig. 1A, grey arrow). The specificity of the Grg3 antibody was validated by its lack of nuclear staining in Grg3−/− embryos and in presence of peptide immunogen on wild type embryos (8; 18). In adult islets, we found that Grg3 was expressed in virtually every insulin+ β cell but was absent from the majority of glucagon+ α cells (wFig. 1B, white arrows). As endocrine maturation proceeds, the percentage of Grg3+ α cells decreases from E17.5 (47%), to the adult (14%), while the percentage of Grg3+ β cells slightly increases from E17.5 (84%), to the adult (98%, Fig. 1C). Thus, Grg3 expression is dynamic during endocrine maturation, marking a large portion of α and β cells early in endocrine development but restricted to the β cell lineage and diminished in α cells as they mature.

In addition, we find that Grg1 protein becomes strongly expressed in adult mouse β cells, but not in the embryonic or neonatal endocrine cells (Suppl. Fig. 1). This suggests that Grg3 plays a non-redundant role during early in endocrine development, but then Grg1 may act redundantly with Grg3 in the adult mouse islet.
We sought to determine if wild type levels of Grg3 in β cells are required for β cell identity and maintenance. The vast majority of Grg3-/- embryos die by E14.5 and mutant embryos that survive have very few endocrine cells (8). Yet, analysis of rare Grg3-/- endocrine cells indicated that they are mainly bihormonal compared to monohormonal endocrine cells of WT littermates (Suppl. Fig. 2). To analyze later stages of endocrine development and maturation, we utilized Grg3+/− mice (8). We found that both E17.5 and 6 week old Grg3+/− pancreata had a decrease in the percentage of insulin+ β cells compared to wild type and an increase in glucagon+ α cells and bihormonal insulin+/glucagon+ cells (Fig. 2A-D), with a variability from islet to islet (Fig. 2E-G). Although we were unable to readily detect changes in Insulin1/2 and Glucagon gene expression in whole Grg3+/− pancreata, interestingly, β cell-specific Glut2 was significantly reduced in Grg3+/− adult pancreata, consistent with a reduction of β cells (Fig. 2H). We also found a significant induction of Somatostatin gene expression in Grg3+/− pancreata (Fig. 2I). 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 (Suppl. Fig. 3). In summary, robust levels of Grg3 are needed to repress α cell identity to maintain the correct ratio of β cells versus 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. 3A). 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
We found that Grg3+/y GFP+ cells express significantly higher levels of the α cell-specific transcription factor Arx (25) and Glucagon (Fig. 3B). However, at E17.5, Insulin1 and Insulin2 were not affected (Fig. 3B). Either Insulin1/2 were not affected because more β cells than α cells are born at this timepoint (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 qRT-PCR on single endocrine cells by sorting E17.5 Ngn3-eGFP;Grg3+/+ or Ngn3-eGFP;Grg3+/y endocrine cells and analyzing gene expression with Fluidigm PCR technology (Fig. 3C), probing for the expression of multiple endocrine genes in each cell. In this assay, we scored the presence of gene expression by determining if a Ct value was achieved. We found that at E17.5, about 92% of Grg3+/+ and 90% of Grg3+/y GFP+ cells were positive for Chga, a marker for the endocrine lineage (27). 64% of Grg3+/+ and 68% of Grg3+/y of the Chga+ endocrine cells expressed Insulin1 and/or Insulin2, indicating that the majority of the endocrine cells sorted from the E17.5 developmental time point are β cells (Fig. 3D). A previous study showed that single Insulin+ β cells can co-express non-β cell hormone genes (28). We analyzed the Insulin+ cells for either monohormonal (Insulin+ only) polyhormonal (Insulin+ plus other hormones expressed) status. We found that of the 14 Insulin+ Grg3+/+ endocrine cells, only one (7%) co-expressed Glucagon and the α cell transcription factor Arx (Fig. 3E). However, in 13 Insulin+ Grg3+/y endocrine cells, 5 cells (38%) co-expressed Glucagon and 3 of those expressed Arx (Fig. 3E). This result recapitulates the change in gene expression of Glucagon and Arx seen in bulk Grg3+/+ versus Grg3+/y endocrine cells (Fig. 3B) and the increase in the bihormonal insulin+/glucagon+ cells in Grg3+/+ pancreata by IF (Fig. 2). Interestingly, the
expression of other non-β endocrine cell hormones (PP, SS and Ghrelin) occurs more frequently in Insulin+ Grg3+/- cells compared to Grg3+/+ cells (Fig. 3E). Overall, the wild type pancreas had more monohormonal Insulin+ cells (9 of 14, 64%) than the Grg3+/- pancreas (3 of 13, 23%), and the Grg3+/- pancreas had more polyhormonal Insulin+ cells (10 of 13, 77%) than the Grg3+/+ pancreas (5 of 14, 36%, p = 0.037, Fig. 3F). 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. This indicates 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 if Grg3 helps maintain the endocrine cell fate in culture. We utilized the mouse cell lines βTC6 (29) and αTC1-6 (30), which express robust levels of Insulin1/2 and Glucagon, respectively (Fig. 4A). We found that Grg3 is expressed higher in βTC6 cells compared to αTC1-6 cells (Fig. 4A). This expression pattern closely resembles the pattern seen in neonates in GFP+ sorted Pdx1-GFP β cells and YFP+ sorted Glucagon-Cre;R26R-YFP α cells (Fig. 4B). To determine if robust levels of Grg3 are required for Glucagon and Arx repression in βTC6 cells, we knocked down Grg3 in βTC6 cells with 2 independent shRNAs introduced by lentivirus (Fig. 4C). In comparison to a control shRNA, the Grg3 shRNAs caused Glucagon expression to increase and Arx expression to be induced (Fig. 4C). These results are similar to the in vivo data with Grg3+/− endocrine cells (Fig. 2, 3).

To determine if 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. 4D). Since
in vivo results suggested that a certain threshold of Grg3 is needed for Glucagon and Arx repression, we investigated if 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 or low GFP-Grg3 (Lo) and high GFP-Grg3 (Hi) expressing cells, we found that Grg3 repressed Glucagon in a dose-dependent manner, but only a high level of Grg3 repressed Arx (Fig. 4E). Interestingly, in long-term cultures high GFP-Grg3 cells significantly induced Insulin2 expression, but not Insulin1 (data not shown), while both low and high GFP-Grg3 cells significantly induced the β cell-specific gene Glut2 (Fig. 4E). This suggests that robust expression of Grg3 represses the α cell program and, over time, allows for the induction of β cell-specific Insulin2 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. 4E) and derepression of Glucagon with reduced Grg3 levels in β cells (Fig. 3B,E; Fig. 4C) suggest that Grg3 also directly represses the Glucagon gene. To test this, we performed chromatin immunoprecipitation (ChIP) on βTC6 and αTC1-6 cells that were infected with viruses encoding GFP and Grg3 fused to a Flag-tag (Suppl. Fig. 4; Flag-Grg3), as performed previously in liver cells (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. 4F). 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 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. 4G). These results, coupled with the functional studies (Fig. 4C-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 co-repressors in the context of neural tube development (34). We found that at E15.5, E17.5, and in neonates, Grg3 was co-expressed with Nkx6.1 in the pancreas (Fig. 5A-C). By performing a Proximity Ligation Assay (PLA) in βTC6 cells to probe for endogenous protein interactions in situ (35), we confirmed that Nkx6.1 and Grg3 interact (Fig. 5D, E). Utilizing *Nkx6.1*+ βTC6 cells and *Nkx6.1*− αTC1-6 cells as a negative genetic control (Fig. 5F), 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. 5G), confirming previously published results (15; 32; 33).

Since Nkx6.1 is not present in α cells to recruit Grg3 to the *Glucagon* promoter, we hypothesized other transcription factors known to be bound to the *Glucagon* promoter in α cells may recruit Grg3. FoxA factors have been shown to directly activate the *Glucagon* gene (36; 37), 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 (Suppl. Fig. 5A-F) and endogenous Grg3 in βTC6 cells (Suppl. Fig. 5G,H) interact with FoxA2. Nkx2.2-Grg3 PLA was performed in βTC6 cells as a positive control (Suppl. Fig. 5I,J), since 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, Grg6). Although we detected *Grg3/TLE3* expression by qRT-PCR, we
were unable to detect nuclear human Grg3/TLE3 (97% homology to mouse) in human islets by IF (Fig. 6A-C). However, of all the family members tested by IF, only Grg1/TLE1 showed specific strong nuclear staining (Fig. 6D-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 α cells (Fig. 6E,G). Also, recently published RNA-seq data of isolated human α and β cells (38) shows that Grg1/TLE1 is enriched 3.35-fold in β cells versus α cells (betacell.org). These findings suggest that human Grg1/TLE1 may play a similar role as mouse Grg3 in repressing α cell-specific genes. To test if 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. 6H). Overall this suggests that Grg1/TLE1 is the predominant Groucho expressed in the human islet, not 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 above studies indicate that Grg3 ultimately helps to promote Insulin expression while repressing α cell-specific genes (Fig. 4E). We determined if ectopic Nkx6.1 and Grg3 co-expression in α cells can help promote robust Insulin expression and help promote α to β cell conversion. Indeed, ectopic Grg3 and Nkx6.1 (Suppl. Fig. 6A,B) could each repress Glucagon and Arx, though more markedly when they are co-expressed (Suppl. Fig. 6C,D). However, only ectopic Nkx6.1 could modestly induce Insulin2 (Suppl. Fig. 6E), while Insulin1 was not detectable in any of the samples (data not shown). This result was not surprising, as Nkx6.1 has been shown to bind the Insulin promoter (32). Interestingly, Insulin2 was also reduced by ectopic Grg3 in these short-term cultures (Suppl. Fig. 6E), however this is most like indirect because Flag-Grg3 did not bind the
Insulin2 promoter (data not shown) and long-term GFP-Grg3 cultures induced Insulin2 (Fig. 4E). We next hypothesized that Grg3 could help promote α to β cell conversion in the presence of Pdx1, a known Insulin inducer (39). Pdx1 has been used in several different contexts to promote β cell conversion (17; 40-42). 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 if insulin was secreted (23; 43).

Ectopic Pdx1 introduced by lentivirus for four days (Fig. 7A,B) modestly repressed Glucagon and failed to repress Arx, but robustly induced Insulin1 and Insulin2 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 Insulin1 and Insulin2 mRNAs (Fig. 7, C-F). Intriguingly, Pdx1 expressed in α cells only allowed for Insulin1 and Insulin2 mRNA expression, but not insulin secretion into the media. By contrast, Grg3 ectopically expressed along with Pdx1 exhibited both Insulin1 and Insulin2 mRNA induction as well as glucose-sensitive insulin secretion (Fig. 7G). Immunofluorescence of αTC1-6 cells expressing GFP, GFP-Grg3, GFP/Pdx1, GFP-Grg3/Pdx1 and βTC6 confirmed Pdx1 protein expression and insulin production (Fig. 7H). 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. 7H). Also of importance, ectopic Grg3 repressed Glucagon protein in both GFP-Grg3 and GFP-Grg3/Pdx1 αTC1-6 cells (Suppl. Fig. 7A) and while inducing Glut2 (Suppl. Fig. 7B). Note, that SS and PP protein was 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 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 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. Our data also indicate that this function is performed by Grg1 in the human islet, not Grg3. This suggests 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; 40; 44), suggesting a pathway to regenerate β cells in diabetics which have reduced 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+ α cells failed to shut off the α cell-specific transcription factor Arx, resulting in the incomplete conversion of α cells into insulin+ β cells (17). Similarly, we found that ectopic Pdx1 expression in αTC1-6 cells induced Insulin1/2 expression but failed to turn off Arx expression. But while we found that co-expression of both Pdx1 and Grg3 induced Insulin1/2 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.
Interestingly, 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. 2011 study (17), the early ectopic Pdx1 expression in newly born Grg3+ endocrine cells allowed for a more efficient conversion towards a β cell identity, while a later induction of Pdx1 in α cells that lack or have low levels of Grg3 may not efficiently convert. Our data suggests 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; 45). This suggests that Grg3 along with other β cell specific transcription factors may be important for the complete conversion towards a functional β cell. Our data indicating that Grg1 is functionally redundant to Grg3 in repressing Glucagon and Arx posits a similar role for Grg1/TLE1 in human β cells. Therefore 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. While 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 co-repressors 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 (36; 37). Our lab 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, our data show that robust levels of Grg3 repress α cell genes.

Our data implies 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 there are fewer insulin+ β cells and more glucagon+ α cells in Grg3+-/ pancreata. Interestingly, Type 2 diabetics have been shown to have reduced levels of insulin with an increase in glucagon (46). Although Grg3+-/ adult mice have normal glucose tolerance, further reduction or impairment of Grg3 repression or metabolic stress may induce a hyperglycemic state. Since 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. Interestingly, a recent study has shown that TLE1 lies near a newly identified Type 2 Diabetes susceptibility locus (47). Further studies are warranted to determine if decreased Grg1/TLE1 expression in human islets results in β to α conversion or increases the risk for developing diabetes.

**Author contributions:** D.E.M. researched the data, C.L., A.S.Z, and A.N. provided human islets, and D.E.M. and K.S.Z. wrote, reviewed, and edited the manuscript. Dr. K. Zaret is the guarantor of this work and, as such, had full access to all the data in the
study and takes responsibility for the integrity of the data and the accuracy of the data analysis. There are no conflicts of interest.

Acknowledgements: D.M. was supported by a postdoctoral fellowship from the University of Pennsylvania (T32 DK007314). The research was supported by a U01DK070430 and a Beckman Research Center/NIDDK/Islet Distribution Center Grant 10028044 to A.N. and a Beta Cell Biology Consortium NIH grant U01DK089571 to K.S.Z.

REFERENCES

1. Spence JR, Wells JM: Translational embryology: using embryonic principles to generate pancreatic endocrine cells from embryonic stem cells. Dev Dyn 2007;236:3218-3227
2. D’Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE: Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006;24:1392-1401
3. Borowiak M, Melton DA: How to make beta cells? Curr Opin Cell Biol 2009;21:727-732
4. Nostro MC, Keller G: Generation of beta cells from human pluripotent stem cells: Potential for regenerative medicine. Semin Cell Dev Biol 2012;23:701-710
5. Turki-Judeh W, Courey AJ: Groucho: a corepressor with instructive roles in development. Curr Top Dev Biol 2012;98:65-96
6. Buscarlet M, Stifani S: The 'Marx' of Groucho on development and disease. Trends Cell Biol 2007;17:353-361
7. Gasperowicz M, Otto F: Mammalian Groucho homologs: redundancy or specificity? J Cell Biochem 2005;95:670-687
8. Metzger DE, Gasperowicz M, Otto F, Cross JC, Gradwohl G, Zaret KS: The transcriptional co-repressor Grg3/Tle3 promotes pancreatic endocrine progenitor delamination and beta-cell differentiation. Development 2012;139:1447-1456
9. Hoffman BG, Zavaglia B, Beach M, Helgason CD: Expression of Groucho/TLE proteins during pancreas development. BMC Dev Biol 2008;8:81
10. Doyle MJ, Loomis ZL, Sussel L: Nkx2.2-repressor activity is sufficient to specify alpha-cells and a small number of beta-cells in the pancreatic islet. Development 2007;134:515-523
11. Papizan JB, Singer RA, Tschen SI, Dhawan S, Friel JM, Hipkens SB, Magnuson MA, Bhushan A, Sussel L: Nkx2.2 repressor complex regulates islet beta-cell specification and prevents beta-to-alpha-cell reprogramming. Genes Dev 2011;25:2291-2305
12. Dhawan S, Georgia S, Tschen SI, Fan G, Bhushan A: Pancreatic beta cell identity is maintained by DNA methylation-mediated repression of Arx. Dev Cell 2011;20:419-429
13. Collombat P, Hecksher-Sorensen J, Krull J, Berger J, Riedel D, Herrera PL, Serup P, Mansouri A: Embryonic endocrine pancreas and mature beta cells acquire alpha and PP cell phenotypes upon Arx misexpression. J Clin Invest 2007;117:961-970
14. Muhr J, Andersson E, Persson M, Jessell TM, Ericson J: Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. Cell 2001;104:861-873
15. Schaffer AE, Taylor BL, Benthuyzen JR, Liu J, Thorel F, Yuan W, Jiao Y, Kaestner KH, Herrera PL, Magnuson MA, May CL, Sander M: Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic Beta cell identity. PLoS Genet 2013;9:e1003274
16. Thorel F, Nepote V, Avril I, Kohno K, Desgraz R, Chera S, Herrera PL: Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 2010;464:1149-1154
17. Yang YP, Thorel F, Boyer DF, Herrera PL, Wright CV: Context-specific alpha- to-beta-cell reprogramming by forced Pdx1 expression. Genes Dev 2011;25:1680-1685
18. Santisteban P, Recacha P, Metzger DE, Zaret KS: Dynamic expression of groucho-related genes Grg1 and Grg3 in foregut endoderm and antagonism of differentiation. Dev Dyn 2010;
19. Lee CS, Perreault N, Brestelli JE, Kaestner KH: Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. Genes Dev 2002;16:1488-1497
20. White P, May CL, Lamounier RN, Brestelli JE, Kaestner KH: Defining pancreatic endocrine precursors and their descendants. Diabetes 2008;57:654-668
21. Herrera PL: Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 2000;127:2317-2322
22. Gu G, Wells JM, Dombkowski D, Preffer F, Aronow B, Melton DA: Global expression analysis of gene regulatory pathways during endocrine pancreatic development. Development 2004;131:165-179
23. Ritz-Laser B, Gauthier BR, Estreicher A, Mamin A, Brun T, Ris F, Salmon P, Halban PA, Trono D, Philippe J: Ectopic expression of the beta-cell specific transcription factor Pdx1 inhibits glucagon gene transcription. Diabetologia 2003;46:810-821
24. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D: Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 1997;15:871-875
25. Collombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Krull J, Gradwohl G, Gruss P: Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes Dev 2003;17:2591-2603
26. Johansson KA, Dursun U, Jordan N, Gu G, Beermann F, Gradwohl G, Grapin-Botton A: Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. Dev Cell 2007;12:457-465
27. Smith SB, Qu HO, Taleb N, Kishimoto NY, Scheel DW, Lu Y, Patch AM, Grabs R, Wang J, Lynn FC, Miyatsuka T, Mitchell J, Seerke R, Desir J, Ejnden SV, Abramowicz M, Kacet N, Weill J, Renard ME, Gentile M, Hansen I, Dewar K, Hattersley AT, Wang R, Wilson ME, Johnson JD, Polychronakos C, German MS: Rfx6 directs islet formation and insulin production in mice and humans. Nature 2010;463:775-780
28. Katsuta H, Akashi T, Katsuta R, Nagaya M, Kim D, Arinobu Y, Hara M, Bonner-Weir S, Sharma AJ, Akashi K, Weir GC: Single pancreatic beta cells co-express multiple islet hormone genes in mice. Diabetologia 2010;53:128-138
29. Poitout V, Stout LE, Armstrong MB, Walseth TF, Sorenson RL, Robertson RP: Morphological and functional characterization of beta TC-6 cells--an insulin-secreting cell line derived from transgenic mice. Diabetes 1995;44:306-313
30. Hamaguchi K, Leiter EH: Comparison of cytokine effects on mouse pancreatic alpha-cell and beta-cell lines. Viability, secretory function, and MHC antigen expression. Diabetes 1990;39:415-425
31. Sekiya T, Zaret KS: Repression by Groucho/TLE/Grg proteins: genomic site recruitment generates compacted chromatin in vitro and impairs activator binding in vivo. Mol Cell 2007;28:291-303
32. Gauthier BR, Gosmain Y, Mamin A, Philippe J: The beta-cell specific transcription factor Nkx6.1 inhibits glucagon gene transcription by interfering with Pax6. Biochem J 2007;403:593-601
33. Schisler JC, Jensen PB, Taylor DG, Becker TC, Knop FK, Takekawa S, German M, Weir GC, Lu D, Mirmira RG, Newgard CB: The Nkx6.1 homeodomain transcription factor suppresses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta cells. Proc Natl Acad Sci U S A 2005;102:7297-7302
34. Muhr J, Andersson E, Persson M, Jessell TM, Ericson J: Groucho-Mediated Transcriptional Repression Establishes Progenitor Cell Pattern and Neuronal Fate in the Ventral Neural Tube. Cell 2001;104:861-873
35. Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, Wester K, Hydbring P, Bahram F, Larsson LG, Langegren U: Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat Methods 2006;3:995-1000
36. Gauthier BR, Schwitzgebel VM, Zaiko M, Mamin A, Ritz-Laser B, Philippe J: Hepatic nuclear factor-3 (HNF-3 or Foxa2) regulates glucagon gene transcription by binding to the G1 and G2 promoter elements. Mol Endocrinol 2002;16:170-183
37. Kaestner KH, Katz J, Liu Y, Drucker DJ, Schutz G: Inactivation of the winged helix transcription factor HNF3alpha affects glucose homeostasis and islet glucagon gene expression in vivo. Genes Dev 1999;13:495-504
38. Dorrell C, Schug J, Lin CF, Canaday PS, Fox AJ, Smirnova O, Bonnah R, Streeter PR, Stoeckert CJ, Jr., Kaestner KH, Grompe M: Transcripts of the major human pancreatic cell types. Diabetologia 2011;54:2832-2844
39. Petersen HV, Serup P, Leonard J, Michelsen BK, Madsen OD: Transcriptional regulation of the human insulin gene is dependent on the homeodomain protein STF1/IPF1 acting through the CT boxes. Proceedings of the National Academy of Sciences 1994;91:10465-10469
40. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA: In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008;455:627-632
41. Gefen-Halevi S, Rachmut IH, Molakandov K, Berneman D, Mor E, Meivar-Levy I, Ferber S: Nkx6.1 promotes PDX-1-induced liver to pancreatic beta-cells reprogramming. Cell Reprogram 2010;12:655-664
42. Mauda-Havakuk M, Litichever N, Chernichovsky E, Nakar O, Winkler E, Mazkereth R, Orenstein A, Bar-Meir E, Ravassard P, Meivar-Levy I, Ferber S: Ectopic PDX-1 expression directly reprograms human keratinocytes along pancreatic insulin-producing cells fate. PLoS One 2011;6:e26298
43. Serup P, Jensen J, Andersen FG, Jorgensen MC, Blume N, Holst JJ, Madsen OD: Induction of insulin and islet amyloid polypeptide production in pancreatic islet glucagonoma cells by insulin promoter factor 1. Proc Natl Acad Sci U S A 1996;93:9015-9020
44. Collombat P, Xu X, Ravassard P, Sosa-Pineda B, Dussaud S, Billestrup N, Madsen OD, Serup P, Heimberg H, Mansouri A: The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. Cell 2009;138:449-462
45. Wilcox CL, Terry NA, Walp ER, Lee RA, May CL: Pancreatic alpha-Cell Specific Deletion of Mouse Arx Leads to alpha-Cell Identity Loss. PLoS One 2013;8:e66214
46. Dunning BE, Gerich JE: The role of alpha-cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications. Endocrine reviews 2007;28:253-283
47. Morris AP, Voight BF, Teslovich TM, Ferreira T, Segre AV, Steinthorsdottir V, Strawbridge RJ, Khan H, Grillert H, Mahajan A, Prokopenko I, Kang HM, Dina C, Esko T, Fraser RM, Kanoni S, Kumar A, Lagou V, Langenberg C, Luan J, Lindgren CM, Muller-Nurasyid M, Pechlivanis S, Rayner NW, Scott LJ, Wiltshire S, Yengo L, Kinnunen L, Rossin EJ, Raychaudhuri S, Johnson AD, Dimas AS, Loos RJ, Vedantam S, Chen H, Florez JC, Fox C, Liu CT, Rybin D, Couper DJ, Kao WH, Li M, Cornelis MC, Kraft P, Sun Q, van Dam RM, Stringham HM, Chines PS, Fischer K, Fontanillas P, Holmen OL, Hunt SE, Jackson AU, Kong A, Lawrence R, Meyer J, Perry JR, Platou CG, Potter S, Rehnberg E, Robertson N, Sivapalaratnam S, Stancakova A, Stirrup K, Thorleifsson G, Tikkkanen E, Wood AR, Almgren P, Atalay M, Benediktsson R, Bonnycastle LL, Burton N, Carey J, Charpentier G, Crenshaw AT, Doney AS, Dorkhan M, Edkins S, Emilsson V, Eury E, Forseen T, Gerton K, Gigante B, Grant GB, Groves CJ, Guiducci C, Herder C, Hreidarsson AB, Hui J, James A, Jonsson A, Rathmann W, Klopp N, Kravic J, Kruitjovk K, Langford C, Leander K, Lindholm E, Lobben S,
Mannisto S, Mirza G, Muhleisen TW, Musk B, Parkin M, Rallidis L, Saramies J, Sennblad B, Shah S, Sigurethsson G, Silveira A, Steinbach G, Thorand B, Trakalo J, Veglia F, Wennauer R, Winckler W, Zabaneh D, Campbell H, van Duijn C, Uitterlinden AG, Hofman A, Sijbrands E, Abecasis GR, Owen KR, Zeggini E, Trip MD, Forouhi NG, Sijbrands E, Eriksson JG, Peltonen L, Nothen MM, Balkau B, Palmer CN, Lyssenko V, Tuomi T, Isomaa B, Hunter DJ, Qi L, Shuldiner AR, Roden M, Barroso I, Wilskaard T, Beilby J, Hovingh K, Price JF, Wilson JF, Rauramaa R, Lakka TA, Lind L, Dedoussis G, Njolstad I, Pedersen NL, Khaw KT, Wareham NJ, Keinanen-Kiukaanniemi SM, Saaristo TE, Korpi-Hyovalti E, Saltevo J, Laakso M, Kuusisto J, Metspalu A, Collins FS, Mohlke KL, Bergman RN, Tuomilehto J, Boehm BO, Gieger C, Hveem K, Cauchi S, Froguel P, Baldassarre D, Tremoli E, Humphries SE, Saleheen D, Danesh J, Ingelsson E, Ripatti S, Salomaa V, Erbel R, Jockel KH, Moebus S, Peters A, Illig T, de Faire U, Hamsten A, Morris AD, Donnelly PJ, Frayling TM, Hattersley AT, Boerwinkle E, Melander O, Kathiresan S, Nilsson PM, Deloukas P, Thorsteinsdottir U, Groop LC, Stefansson K, Hu F, Pankow JS, Dupuis J, Meigs JB, Altshuler D, Boehnke M, McCarthy MI: Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. Nat Genet 2012;44:981-990

FIGURE LEGENDS

Fig. 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 (grey) on the same section showing most insulin+ cells are Grg3+ (red arrows, red box) while many glucagon+ cells are Grg3- or have low level Grg3 expression (grey arrows, grey box). B. Immunofluorescence for Grg3, insulin and glucagon in adult islets, showing virtually all insulin+ cells are Grg3+ while glucagon+ cells are Grg3- (white arrows). C. The percent of Grg3+ β cells and α cells were counted at E17.5 (n=4 animals, 588 α cells and 932 β cells analyzed) and adulthood (n=14 islets from 3 animals, 547 α cells and 1479 β cells analyzed). * = p < 0.0001

Fig. 2. Grg3+- pancreata have less β cells and more α cells than Grg3+/. A. E17.5 Grg3+/+ and Grg3+/- pancreata stained for insulin and glucagon. Yellow arrow points out a double positive (insulin+/glucagon+) cell in the Grg3+/- pancreas. B. Percentage of insulin+ β cells, glucagon+ α cells and double positive cells in Grg3+/+
(n=4 animals, 1402 cells analyzed) and Grg3+/− (n=4 animals, 2000 cells analyzed) E17.5 pancreata. C. Adult Grg3+/+ and Grg3+/− islets stained for insulin and glucagon. Indicated boxes are enlarged to show single insulin or glucagon channel. D. Percentage of insulin+ β cells, glucagon+ α cells and double positive cells in Grg3+/+ (n=3 animals, 2551 cell analyzed) and Grg3+/− adult islets (n=3 animals, 3264 cells analyzed). The percentage of Glucagon+ (E), Insulin+ (F) and Glucagon/Insulin+ (G) cells in individual islets. Glut2 expression was significantly reduced in adult Grg3+/− pancreata (H), while Somatostatin expression increased. # = p = 0.053, * = p < 0.05, ** = p < 0.01.

**Fig. 3. Robust Grg3 expression helps 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 Insulin1 and Insulin2 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%) express Insulin in Grg3+/+ and Grg+/− pancreata, respectively. E. Grg3+/+ and Grg3+/− Insulin1 and/or Insulin2+ cells were analyzed for Glucagon, Arx, Pancreatic Peptide (PP), Somatostatin (SS) and Ghrelin positivity. Filled in boxes represent a positive call for the indicated gene in the indicated cell. F. Grg3+/+ has more monohormonal Insulin+ cells (9 of 14, 64%) than the Grg3+/− (3 of 13, 23%), and has fewer polyhormonal Insulin+ (5 of 14, 36%) cells than Grg3+/− (10 of 13, 77%); one-tailed Fisher’s exact test, p = 0.037.
Fig. 4. Grg3 directly represses Glucagon and Arx. A. αTC1-6 and βTC6 cells were analyzed for Grg3, Glucagon, Insulin1 and Insulin2. B. YFP+ α cells sorted from Glucagon<sup>Cre</sup>, R26R<sup>YFP</sup> neonates and GFP+ β cells sorted from Pdx1<sup>GFP</sup> neonates were analyzed for Grg3, Glucagon, Insulin1 and Insulin2. 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) via lentiviral infection for 4 days in αTC1-6 cells repressed Glucagon and Arx compared to GFP infected cells. E. GFP-Grg3 was stably expressed for 3 weeks in αTC1-6 cells and sorted for negative GFP expression, low GFP and high GFP, and Grg3 expression increased with GFP intensity. Glucagon was repressed in a dose-dependent manner while only the highest Grg3 dose repressed Arx and induced Insulin2. F. βTC6 cells were infected with GFP or Flag-Grg3, and ChIP was performed using an αFlag antibody. qPCR of the Glucagon, Hnf1β and Hnf6 promoters indicate that Flag-Grg3 occupies the Glucagon promoter, while Hnf1β and Hnf6 promoters are not bound. G. αTC1-6 cells were infected with GFP or Flag-Grg3, and ChIP was performed using an αFlag antibody. Similarly, qPCR of the Glucagon promoter shows that it is occupied in Flag-Grg3 cells. Each data point was repeated 2 to 4 times.

Fig. 5. Grg3 is recruited to the Glucagon promoter via a Nkx6.1 interaction. A-C. Immunofluorescence of Nkx6.1 and Grg3 on E15.5, E17.5 and neonatal pancreata. Yellow arrows indicate coexpressing cells. D. Proximity Ligase Assay 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
low nonspecific signal; immunofluorescence indicates the specificity of the Grg3 and IgG antibodies. F. Grg3 is expressed much more abundantly in βTC6 cells versus 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.

Fig. 6. Grg1 is the predominant Groucho-family member expressed in human islets. A,B. Nuclear Grg3 is not detectable in human islets (A) and non-specific 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 alpha cells (white arrows) compared to 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 below) indicate that Grg1 staining in β cells is significantly more intense than in α cells (G). mGrg1 is more abundant in βTC6 cells than αTC1-6 cells (H). I. αTC1-6 were transfected with GFP fused to Grg1 (GFP-Grg1) via transfection for 3 days and cells sorted based of GFP intensity. Ectopic GFP-Grg1 repressed Glucagon and Arx in a dose dependant manner in αTC1-6 cells.

Fig. 7. Grg3 works with Pdx1 to repress α cell genes and induce glucose-stimulated insulin secretion. A-B. GFP-Grg3 was coexpressed with Pdx1 in αTC1-6 cells for 1 week. C-F. Pdx1 modestly repressed Glucagon and failed to repress Arx but induced abundant levels of Insulin1 and Insulin2. GFP-Grg3 coexpressed with Pdx1 represses both Glucagon and Arx while inducing Insulin1 and Insulin2. G. αTC1-6 cells
ectopically expressing GFP, GFP-Grg3 or Pdx1 failed to secrete insulin, but GFP-Grg3 coexpressed with Pdx1 induced 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 were 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 while GFP and GFP-Grg3 cells do not. GFP-Grg3/Pdx1 αTC1-6 and βTC6 cells are positive for C-peptide while GFP/Pdx1, GFP and GFP-Grg3 αTC1-6 cells are not. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.


**Metzger/Zaret Figure 3**

A. *Ngn3-eGFP; Grg3+/+* or *Ngn3-eGFP; Grg3+-/−* E17.5 pancreas

FACS bulk GFP+ cells

RT-qPCR gene expression

B. 

|Gene| Arx| Glucagon| Insulin 1| Insulin 2|
|---|---|---|---|---|
|Grg3+/+| p=0.008| p=0.017| | |
|Grg3+-/−| |

B. Relative Expression Normalized to *Gapdh*

C. *Ngn3-eGFP; Grg3+/+* or *Ngn3-eGFP; Grg3+-/−* E17.5 pancreas

FACS individual GFP+ cells

Fluidigm qPCR gene expression of single cells

D. Endocrine cells

|# of cells| Insulin+| Chga+|
|---|---|---|
|Grg3+/+| |
|Grg3+-/−| |

E. *Insulin*+ cells

F. *Insulin*+ cells

Metzger/Zaret Figure 3
Metzger/Zaret Figure 4

A Cell lines

| Grg3 | Glucagon | Insulin1 | Insulin2 |
|------|----------|----------|----------|
| αTC  | βTC      | αTC      | βTC      |

B Sorted Endocrine Cells

| Sorted Population: |
|-------------------|
| βTC6              |

C βTC6

D αTC1-6 (4 day culture)

E αTC1-6 (3 week culture)

F βTC ChIP: αFlag

G αTC ChIP: αFlag
Metzger/Zaret Figure 5

(A) DAPI

(B) DAPI

(C) DAPI

(D) Grg3/Nkx6.1 PLA

(E) Grg3/IgG PLA

(F) Relative Expression Normalized to Gapdh

(G) ChIP: αNkx6.1

Hnf6 Glucagon

ChIP/Input (relative units)

αTC βTC

Promoter: Hnf6 Glucagon
Metzger/Zaret Figure 6

A. Human Islets

- Insulin
- Grg3
- DAPI

B. Insulin
- Grg3
- +pep
- DAPI

C. RT-PCR of Human Islets

- Gapdh
- Grg3

D. Insulin
- Grg1
- DAPI

E. Gluc
- Grg1
- DAPI

F. Insulin
- Grg1
- +pep
- DAPI

G. Gluc/Grg1
- Ins/Grg1

- Human Grg1 intensity

- p<0.0001

H. Grg1

- Relative Expression Normalized to Gapdh

I. αTC1-6 cells

- Grg1
- Glucagon
- Arx

- Sorted Population:
  - GFP-Grg1
  - Neg Lo
  - Hi

- αTC
- pTC

- p<0.01
- p<0.001
- p<0.0001
- p<0.005
- p<0.005
Metzger/Zaret Figure 7

A) Relative Expression Normalized to Gapdh

B) Relative Expression Normalized to Gapdh

C) Glucagon

D) Arx

E) Insulin1

F) Insulin2

G) αTC1-6 Insulin Secretion

H) αTC1-6

**βTC6**
Supplemental Figures for Diabetes 13-0867, Metzger/Zaret

Suppl. Fig. 1. Grg1 is expressed in the adult mouse islet. Pancreata were co-stained for Grg1 and Insulin (A-C). Grg1 did not stain the insulin+ β cells in E17.5 embryos (A) or neonatal pups (B), but Grg1 was robustly expressed in adult mouse islets (C).
Suppl. Fig. 2. E14.5 Grg3/- embryos have bihormonal endocrine cells. A,B. Triple staining with Insulin, Glucagon and Pdx1 showing monohormonal Insulin+ and Glucagon+ cells in E14.5 Grg+/+ (A), while one rare surviving E14.5 Grg3/- has a few endocrine cells that are bihormonal (B). Yellow arrow shows bihormonal cells while green and red arrows indicate unihormonal Insulin+ and Glucagon+ cells, respectively.
Suppl. Fig. 3. *Grg3*+/− adult mice have normal glucose tolerance tests. 6 week old (A) and 12 month old (B) *Grg3*+/+ and *Grg3*+/− were subjected to glucose tolerance tests. Both *Grg3*+/+ and *Grg3*+/− had normal glucose tolerance curves. Error bars denote SEM. Animals were males and females, the latter never having been pregnant.
Suppl. Fig. 4. Flag-Grg3 lentiviral infection of βTC6 and αTC1-6 cells. A. Western blot of βTC6 cells infected with or without Flag-Grg3 and probed for Grg3, Flag and Gapdh. B. αTC1-6 cells infected with GFP or Flag-Grg3 and stained by immunofluorescence for Flag. GFP signal is the natural fluorescence from the protein.
Suppl. Fig. 5. Grg3 interacts with FoxA2 in α and β cells. A-I. Proximity Ligase Assays (PLA) were performed on αTC1-6 and βTC6 cells probing for Grg3/Foxa2 interaction. GFP-Grg3 ectopically expressed in αTC1-6 cells interacts with FoxA2 (A) to a greater extent than endogenous levels of Grg3 (B). Controls using Grg3 and IgG show little nonspecific signal (C,D). Immunofluorescence of indicated antibodies shows antibody specificity (E-F). Endogenous Grg3 and FoxA2 interact in βTC6 cells (G), and immunofluorescence indicates antibody specificity (H). Grg3 and Nkx2.2 also interact in βTC6 cells (I), and immunofluorescence shows antibody specificity (J).

(please see next page for images)
Suppl. Fig. 5. Grg3 interacts with FoxA2 in α and β cells.
**Suppl. Fig. 6.** Grg3 works with other transcription factors to repress α cell genes.

A, B. GFP-Grg3 and Nkx6.1 were ectopically expressed in αTC1-6 cells for 1 week. C, D. Grg3 and Nkx6.1 both repress *Glucagon* and *Arx* and coexpression of both factors repress even more. E. Grg3 and Grg3/Nkx6.1 infection failed to induce *Insulin2* expression, but Nkx6.1 by itself modestly induced *Insulin2*. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.
Suppl. Fig. 7. **Grg3 represses Glucagon in αTC1-6 cells and induced Glut2 expression.** A. Ectopic GFP-Grg3 represses Glucagon protein in αTC1-6 cells without and with co-introduction with Pdx1. B. GFP-Grg3 also induces Glut2 expression in αTC1-6 cells infected with Pdx1.