Loss of mTORC1 signaling alters pancreatic α cell mass and impairs glucagon secretion

Nadejda Bozadjieva, …, Patrick E. MacDonald, Ernesto Bernal-Mizrachi


Glucagon plays a major role in the regulation of glucose homeostasis during fed and fasting states. However, the mechanisms responsible for the regulation of pancreatic α cell mass and function are not completely understood. In the current study, we identified mTOR complex 1 (mTORC1) as a major regulator of α cell mass and glucagon secretion. Using mice with tissue-specific deletion of the mTORC1 regulator Raptor in α cells (αRaptorKO), we showed that mTORC1 signaling is dispensable for α cell development, but essential for α cell maturation during the transition from a milk-based diet to a chow-based diet after weaning. Moreover, inhibition of mTORC1 signaling in αRaptorKO mice and in WT animals exposed to chronic rapamycin administration decreased glucagon content and glucagon secretion. In αRaptorKO mice, impaired glucagon secretion occurred in response to different secretagogues and was mediated by alterations in KATP channel subunit expression and activity. Additionally, our data identify the mTORC1/FoxA2 axis as a link between mTORC1 and transcriptional regulation of key genes responsible for α cell function. Thus, our results reveal a potential function of mTORC1 in nutrient-dependent regulation of glucagon secretion and identify a role for mTORC1 in controlling α cell–mass maintenance.

Find the latest version:

https://jci.me/90004/pdf
Loss of mTORC1 signaling alters pancreatic α cell mass and impairs glucagon secretion

Nadejda Bozadjieva,1,2 Manuel Blandino-Rosano,1,3 Jennifer Chase,2 Xiao-Qing Dai,4 Kelsey Cummings,1 Jennifer Gimeno,3 Danielle Dean,1 Alvin C. Powers,5,6,7 George K. Gittes,4 Markus A. Rüegg,3 Michael N. Hall,9 Patrick E. MacDonald,4 and Ernesto Bernal-Mizrachi1,2,3,10

1Department of Internal Medicine, Division of Metabolism, Endocrinology and Diabetes, and 2Graduate Program in Cellular and Molecular Biology, University of Michigan, Ann Arbor, Michigan, USA. 3Department of Internal Medicine, Division of Gastroenterology and Hepatology, University of Miami, Miami, Florida, USA. 4Alberta Diabetes Institute and Department of Pharmacology, Edmonton, Alberta, Canada. 5Department of Medicine, Division of Diabetes, Endocrinology, and Metabolism, and 6Department of Molecular Physiology & Biophysics, Vanderbilt University, Nashville, Tennessee, USA. 7VA Tennessee Valley Healthcare, Nashville, Tennessee, USA. 8Children’s Hospital, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. 9Biozentrum, University of Basel, Basel, Switzerland. 10Veterans Affairs Medical Center, Miami, Florida, USA.

Glucagon plays a major role in the regulation of glucose homeostasis during fed and fasting states. However, the mechanisms responsible for the regulation of pancreatic α cell mass and function are not completely understood. In the current study, we identified mTOR complex 1 (mTORC1) as a major regulator of α cell mass and glucagon secretion. Using mice with tissue-specific deletion of the mTORC1 regulator Raptor in α cells (αRaptorKO), we showed that mTORC1 signaling is dispensable for α cell development, but essential for α cell maturation during the transition from a milk-based diet to a chow-based diet after weaning. Moreover, inhibition of mTORC1 signaling in αRaptorKO mice and in WT animals exposed to chronic rapamycin administration decreased glucagon content and glucagon secretion. In αRaptorKO mice, impaired glucagon secretion occurred in response to different secretagogues and was mediated by alterations in KATP channel subunit expression and activity. Additionally, our data identify the mTORC1/FoxA2 axis as a link between mTORC1 and transcriptional regulation of key genes responsible for α cell function. Thus, our results reveal a potential function of mTORC1 in nutrient-dependent regulation of glucagon secretion and identify a role for mTORC1 in controlling α cell–mass maintenance.

Introduction

Type 1 (T1D) and type 2 diabetes (T2D) are characterized by uncontrolled hyperglycemia associated with the progressive decrease in insulin. Glucagon, insulin’s counterregulatory hormone, plays a major role in maintaining homeostasis by promoting glucose production via hepatic glycogenolysis and gluconeogenesis. Glucagon levels are elevated in insulin-resistant/nondiabetic T1D and T2D patients, leading to enhanced hepatic glucose output and thereby exacerbating hyperglycemia (1–3). On the contrary and much less understood is the failure of α cells to secrete glucagon in response to hypoglycemia. This presents a major limiting factor for optimal glucose control in T1D diabetes or advanced T2D patients (4–6). Thus, a better understanding of the molecular mechanisms governing glucagon levels could have major implications in understanding abnormal responses to hypoglycemia in diabetes and provide novel avenues for diabetes management.

Mice with loss of the insulin receptor in α cells have increased fed glucagon levels, suggesting that insulin signaling mediates the suppression of glucagon secretion in the fed state (7). In addition, mice with pancreatic deletion of the insulin receptor substrate 2 (IRS2) exhibit decreased α cell mass and lower glucagon protein and RNA levels, implicating IRS signaling in the control of α cell mass and glucagon expression (8). Insulin inhibits glucagon gene transcription and secretion and promotes α cell proliferation by activation of IRS2/Pi3K/Akt signaling (9, 10). α Cell proliferation is reduced by treatment with the mTOR complex 1 (mTORC1) inhibitor rapamycin, suggesting that downstream of the insulin receptor mTORC1 mediates the effects of insulin on α cell mass and glucagon secretion (11, 12). The amino acids arginine, alanine, and glutamine potentiate glucagon secretion and this effect is suppressed by high glucose in an insulin-independent manner (13). Recent studies demonstrate that interruption of glucagon receptor signaling by genetic inactivation or treatment with small molecules or glucagon receptor antibodies increases amino acid availability and leads to increased α cell proliferation in an mTOR-dependent manner (12, 14–17). These findings support the concept that α cell mass and glucagon secretion are sensitive to extracellular signals including nutrients (amino acids, glucose) and growth factors (insulin) and that the mTORC1 pathway may be involved as a downstream regulator of one or both of these processes. However, how downstream targets of nutrient or insulin receptor signaling regulate α cell mass and glucagon secretion in vivo is currently unknown.

To investigate the importance of endogenous mTORC1 function in α cell mass and glucagon secretion, we generated mice with tissue-specific deletion of Raptor in α cells. Our data uncovered

Conflict of Interest: The authors have declared that no conflict of interest exists.
License: This work is licensed under the Creative Commons Attribution 4.0 International License. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.
Submitted: August 16, 2016; Accepted: September 26, 2017.
transcription of critical α cell genes. This work provides insights into how nutrient-dependent glucagon secretion and α cell mass are regulated and suggest that pharmacologic inhibition of this pathway using immunosuppressant medications, such as everolimus or rapamycin, could alter glucagon levels and glucose homeostasis.

Results

Lack of mTORC1 signaling after deletion of Raptor in α cells. α Cell–specific deletion of Raptor was achieved by crossing glucagon-Cre and Raptor floxed mice (αRaptor KO) (18, 19). Deletion of flanked exon 6 exclusively in α cells from αRaptor KO mice was demonstrated by nested reverse transcription PCR (RT-PCR) for exon 6 using different tissues and single α cells (Figure 1A) (19). Loss of mTORC1 signaling was confirmed by lack of phospho-S6 (Ser240) immunofluorescence staining only in glucagon-positive cells in dispersed islets from 1-month-old αRaptor KO mice (Figure 1B). To validate the reduction in mTORC1 signaling in α cells from αRaptor KO mice, we assessed phospho-S6 (Ser240), glucagon, and insulin staining in dispersed islets by flow cytometry using quantitative mean fluorescence intensity (MFI). Figure 1C shows pS6 MFI levels in α cells (glucagon+ cell count) and Figure 1D includes pS6 MFI levels in β cells (insulin+ cell count). Phospho-S6 (Ser240) levels were nearly lost in glucagon-positive cells from αRaptor KO mice (red curve) compared with controls (black curve) (Figures 1C). In contrast, the MFI for phospho-S6 (Ser240) was similar in insulin-positive cells from αRaptor KO mice (red curve) and controls (black curve) (Figure 1D). Recombination efficiency of glucagon-Cre assessed by crossing these mice to reporter mice showed that Cre-mediated recombination was achieved in the majority of α cells (84.2% ± 6.4%, n = 4). We also report glucagon-Cre recombination in neurons of the nucleus of the solitary tract (nucleus tractus solitarius, NTS) (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI90004DS1).

Low fed and fasting glucagon levels in mice with loss of mTORC1 signaling in α cells. Body weight and random-fed blood glucose were not different between control mice and αRaptor KO or αRaptor HET (glucagon-Cre; Raptor floxed) mice (Figure 2, A and B). Glucose tolerance at 2 and 8 months of age was not different between the 3 groups (Figure 2, C and G). Control mice exhibited a decrease in glucose levels after 12 hours of fasting at both 2 and 8 months of age (Figure 2, D and H). In contrast, 2-month-old αRaptor KO mice were able to maintain blood glucose levels during the first 12 hours of fasting and glucose almost returned to the levels of control mice at 24 hours (Figure 2D and
αRaptorKO and αRaptorHET mice (Figure 2, E and I). No differences were observed in fed or fasting insulin levels between controls, αRaptorKO, and αRaptorHET mice at 2 and 8 months (Figure 2, F and J). Finally, assessment of GLP-1 levels showed no difference in fed or fasting circulating active GLP-1 between αRaptorKO, αRaptorHET, and controls at 2 months of age (Supplemental Figure 3A). Active GLP-1 levels in intestinal extracts from 2-month-old mice showed no differences between control and αRaptorKO mice, suggesting that intestinal enteroendocrine cells expressing GLP-1 were conserved, perhaps due to mosaic Cre-mediated recombination in intestinal stem cells (Supplemental Figure 3B).

mTORC1 signaling is necessary for the maintenance of postnatal α cells. Morphometric analysis at postnatal day 1 (newborn) demonstrated that αRaptorKO mice were born with normal α cell mass (Figure 3, A and B, α cells depicted with white arrows). At 2 weeks of age, αRaptorKO mice exhibited normal α cell mass, lower levels of proliferation (assessed by Ki67), and no changes in apoptosis (assessed by TUNEL) (Figure 3C and Supplemental Figure 4, A and B). αRaptorKO mice displayed loss of α cells as evidenced by a reduction in α cell mass starting at 1 month of age (Figure 3, A and D; α cells depicted with white arrows in 3A). Flow cytometric

---

Figure 2. Mice with loss of mTORC1 signaling in α cells exhibit low fed and fasting glucagon levels. (A) Body weight and (B) random-fed blood glucose levels in control, αRaptorKO, and αRaptorHET mice (n = 9). (C) Intraperitoneal glucose tolerance test in 2-month-old (n = 3–4) and (G) 8-month-old control, αRaptorKO, and αRaptorHET mice (n = 5–8). (D) Fasting blood glucose in 2-month-old (n = 7–9) and (H) 8-month-old (n = 3–4) control, αRaptorKO, and αRaptorHET mice. (E) Fed and fasted glucagon levels in 2-month-old (n = 5–6) and (I) 8-month-old (n = 5–6) control, αRaptorKO, and αRaptorHET mice. (F) Fed and fasted insulin levels in 2-month-old (n = 3–4) and (J) 8-month-old mice (n = 4). Data are shown as means ± SEM. *P ≤ 0.05 (1-way ANOVA with Dunnett’s post-test).
Figure 3. mTORC1 signaling is necessary for maintenance of postnatal α cells. (A) Immunofluorescent staining for insulin and glucagon in pancreatic sections from control, αRaptorKO, and αRaptorHET mice (α cells depicted with white arrows). Scale bars: 50 μm. (B) Quantification of α cell fraction at postnatal day 1 (newborn) control, αRaptorKO, and αRaptorHET mice (n = 3). (C) Quantification of α cell mass at 2 weeks (n = 4). (D) 1-month-old (n = 4), and (E) 2-month-old control, αRaptorKO, and αRaptorHET mice (n = 4–5). (F) Pancreatic glucagon content (n = 4–6) in 2-month-old mice. (G) Quantification of α cell mass (n = 3–4) and (H) pancreatic glucagon content (n = 3–4) in 8-month-old mice. (I) Quantification of β cell fraction at postnatal day 1 (newborn) (n = 3) and (J) β cell mass in 2-month-old mice (n = 4–5). (K) Electron microscopy of α cells from 1-month-old control and αRaptorKO mice. Scale bars: 800 nm (control) and 600 nm (αRaptorKO). (L) Quantification of α cell size by morphometric analysis in control and αRaptorKO mice at 1 month of age (n = 3). (M) Analysis of glucagon content by flow cytometric analysis in dispersed α cells from control and αRaptorKO mice at 3 weeks of age (n = 3–4). MFI, mean fluorescence intensity. (N) Quantification of GFP-LC3 puncta and representative images of dispersed α cells from 3-week-old control and αRaptorKO mice (n = 50 cells) crossed to an in vivo reporter of autophagy (GFP-LC3 mice). Scale bars: 10 μm. Data for C, D, L, and N are shown as means ± SEM. *P ≤ 0.05 (1-way ANOVA with Dunnett’s post-test).

Analysis showed that the reduction of α cell mass at 1 month of age resulted from increased apoptosis (assessed by annexin V) and a trend towards decreased proliferation (assessed by Ki67) (Supplemental Figure 4, C and D). α Cell mass progressively decreased in αRaptorKO mice at 2 and 8 months (Figure 3, A, E, and G). A progressive decline in total pancreatic glucagon content was also observed at 2 and 8 months (Figure 3, F and H). In contrast, β, δ (somatostatin), and pancreatic polypeptide (PP) cell mass were not altered in 2-month-old αRaptorKO mice (Figure 3, I and J and Supplemental Figure 4, E–G). α Cell mass and pancreatic glucagon content were comparable between controls and in αRaptorHET mice at 2 months of age (Figure 3, E and F). However, by 8 months αRaptorHET mice showed a reduction in α cell mass and pancreatic glucagon content (Figure 3, G and H). The number of α cells in older αRaptorHET mice assessed by flow cytometry was reduced, confirming these results (Supplemental Figure 4). However, cell size and cellular glucagon content measured by flow cytometry was unaltered in the remaining α cells of αRaptorHET mice (Supplemental Figure 4, D and E). Electron microscopy in 1-month-old αRaptorKO showed a reduction in α cell size that was confirmed by cell size measurements (Figure 3, K and L). In addition, the number of glucagon granules appeared reduced in α cells from αRaptorKO mice and this was independently validated by reduced intracellular glucagon levels using flow cytometry (Figure 3M).

Given the known role of mTORC1 in autophagy, we designed experiments to examine the contribution of autophagy to the loss of α cells in αRaptorKO mice by crossing to an in vivo reporter of autophagy (GFP-LC3 mice) (20). These studies showed that αRaptorKO exhibited increased GFP-LC3 puncta in α cells, consistent with the presence of increased autophagy (Figure 3N). Taken together, the results of these studies suggest that reduction in α cell mass between 2 and 4 weeks results from a combination of decreased proliferation and increased apoptosis.

αRaptorHET mice have decreased glucagon responses to hypoglycemia and glucoprivic conditions. The lower fasting glucagon levels with concomitant normal α cell mass in 2-month-old αRaptorHET mice suggested that these mice exhibited a defect in glucagon secretion. To further investigate the role of mTORC1 in glucagon secretion, we subjected these mice to different stimulatory conditions in vivo. Examination of glucagon secretion by insulin-induced hypoglycemia in 2-month-old mice showed that insulin induced similar decreases in blood glucose in αRaptorKO, αRaptorHET, and control mice (Figure 4A). However, glucagon secretion after insulin-induced hypoglycemia was compromised in αRaptorKO mice and this was likely explained by the severe loss of α cell mass (Figure 4B). In contrast, αRaptorHET mice were able to respond, but showed reduced glucagon secretion at 30 minutes after insulin injection (Figure 4B). Next, we examined glucagon secretion under glucoprivic signals induced by 2-deoxy-D-glucose (2DG) injection, a nonmetabolizable glucose analog that inhibits phosphorylation of glucose by hexokinase (Figure 4C). 2DG administration raises blood glucose by increases in counterregulatory hormones (epinephrine, corticosterone, and glucagon) and hepatic glucose output. Assessment of glucagon secretion after 2DG injection showed that αRaptorKO failed to respond to glucoprivic conditions, a defect that was likely due to severe loss of α cell mass (Figure 4D). αRaptorHET mice displayed impaired glucagon responses compared with the controls at 30 minutes after 2DG injection (Figure 4D). Interestingly, αRaptorHET mice had a higher response in blood glucose output at 30 minutes, suggesting that compensatory counterregulatory responses could be enhanced in conditions of chronic low glucagon (Figure 4D). These data further showed that αRaptorHET mice have a defect in glucagon secretion.

Rapamycin treatment in vivo inhibits glucagon secretory responses to hypoglycemia and reduces glucagon content in isolated islets. To validate the alterations in glucagon secretion observed in αRaptorHET mice, we assessed the effect of pharmacologic inhibition of mTORC1 by intraperitoneal administration of rapamycin to wild-type mice every other day for total of 5 injections (Figure 5A). Body weight and fed blood glucose were not affected by rapamycin or vehicle treatment (Figure 5, B and C). After insulin-induced hypoglycemia, rapamycin-treated mice displayed lower glucose at 120 minutes after insulin injection, suggesting a decrease in counterregulatory responses to hypoglycemia (Figure 5D). Evaluation of the glucagon response in these mice revealed that glucagon secretory response to hypoglycemia was blunted (Figure 5E) and glucagon content in isolated islets was reduced in rapamycin-treated mice (Figure 5F). In order to avoid confounding factors that result from systemic actions of rapamycin, we measured glucagon secretion in wild-type islets after acute exposure to rapamycin. In these studies, wild-type islets were preincubated with 30 nM rapamycin for 30 minutes followed by a 2-hour incubation with different glucose concentrations from 1 mM to 24 mM. As expected, glucagon secretion decreased with higher glucose concentrations in untreated control islets (Figure 5G). However, 30 minutes of rapamycin treatment decreased glucagon secretion at 1 mM glucose, confirming in vivo data showing that rapamycin inhibits glucagon secretion in response to hypoglycemia (Figure 5G). In addition, glucagon secretion was suppressed by increasing glucose conditions and this effect was not altered by rapamycin. The reduction in glucagon secretion induced by acute treatment with rapamycin was not explained by alterations in glucagon content (Figure 5H), suggesting that short-term rapamycin treatment can inhibit glucagon secretion induced
by low glucose independently of alterations in glucagon content. Similarly, glutamine also failed to stimulate glucagon secretion in wild-type islets also treated acutely with rapamycin (30 nM), and these changes were independent of glucagon content (Supplemental Figure 5, A and B).

**Glucagon secretory responses induced by pharmacologic manipulation of K\textsubscript{ATP} channels in \(\alpha\)Raptormice is abnormal.** To uncover the mechanisms responsible for impaired glucagon secretion in \(\alpha\)Raptor mice, we examined secretory responses of isolated islets. Glucagon secretion in response to depolarization induced by potassium chloride (KCl; 30 mM) was similar between control islets. Glucagon secretion in response to depolarization induced by potassium chloride (KCl; 30 mM) was similar between control islets (Figure 6A). Next, we examined the glucagon response to arginine, a secretagogue that induces glucagon secretion in part by modulating K\textsubscript{ATP} channels (21). Glucagon secretion induced by arginine was blunted in \(\alpha\)Raptor islets (Figure 6B). Taken together, these data suggested that the defect in glucagon secretion in \(\alpha\)Raptor islets resides in steps prior to cell depolarization and possibly at the level of the K\textsubscript{ATP} channel. We further evaluated glucagon secretion during pharmacological modulation of K\textsubscript{ATP} channel activity with increasing concentrations of tolbutamide (K\textsubscript{ATP} channel antagonist) and diazoxide (K\textsubscript{ATP} channel agonist). Under low-glucose conditions, when a relatively larger fraction of K\textsubscript{ATP} channels are open, tolbutamide treatment of control islets led to glucagon secretion up to concentrations of 10 \(\mu\)M, whereas glucagon secretion was inhibited at higher concentrations, as previously described (22–24). In contrast, tolbutamide treatment of \(\alpha\)Raptor islets failed to induce glucagon secretion and suppressed glucagon release at lower concentrations of tolbutamide (Figure 6C). Next we tested the effects of diazoxide-mediated opening of K\textsubscript{ATP} channels at high glucose, where most K\textsubscript{ATP} channels should be closed. Increasing concentrations of diazoxide (0–10 \(\mu\)M) relieved the suppression of glucagon secretion in control islets (Figure 6D). In contrast, islets from \(\alpha\)Raptor mice were unresponsive to diazoxide and this agent failed to induce glucagon secretion (Figure 6D). Conversely, under low-glucose (1 mM) conditions when at least some K\textsubscript{ATP} channels are open, diazoxide further induced glucagon secretion at 1 \(\mu\)M followed by a dose-dependent suppression at 10 and 100 \(\mu\)M in control islets (Figure 6D). Under this condition, \(\alpha\)Raptor islets still failed to respond to diazoxide (Figure 6, D and E). Taken together, these studies suggested that K\textsubscript{ATP} channel function is altered in \(\alpha\) cells with reduced mTORC1 signaling.

**mTORC1 positively regulates glucagon secretion by modulating K\textsubscript{ATP} channel expression.** Next, we tested whether the changes in glucagon secretion obtained by pharmacologic manipulation of K\textsubscript{ATP} channels in \(\alpha\)Raptor mice resulted from alterations in K\textsubscript{ATP} channel activity. Whole-cell patch clamp was performed on \(\alpha\) cells at low glucose from Glucagon-Cre;tdTomato and \(\alpha\)Raptor;tdTomato mice expressing a fluorescent reporter in \(\alpha\) cells. Following establishment of the whole-cell patch clamp, and subsequent washout of intracellular ATP, K\textsubscript{ATP} channel current was significantly reduced in \(\alpha\) cells compared with controls (Figure 6, F and G). To test the hypothesis that these results could be explained by alterations in K\textsubscript{ATP} subunit expression, we measured the expression of K\textsubscript{ATP} channels subunits Kir6.2 and sulfonylurea receptor subunit 1 (SUR1) in \(\alpha\) cells. We isolated an enriched population of \(\alpha\) cells from \(\alpha\)Raptor;tdTomato mice using FACS using dispersed islets from \(\alpha\)Raptor;tdTomato and control mice crossed to Ins1-EGFP and CAG-tdTomato reporter mice (refer to Methods and Supplemental Figure 6). Our data showed that the enriched \(\alpha\) cell population from \(\alpha\)Raptor mice had decreased SUR1 and Kir6.2 mRNA expression (Figure 6H). Supporting these findings, single-cell mRNA expression for SUR1 in \(\alpha\) cells also showed that young \(\alpha\)Raptor;tdTomato mice had fewer \(\alpha\) cells positive for SUR1 (Figure 6I). Unfortunately, we were unable to detect expres-
sion of Kir6.2 in single cells using this methodology. Lastly, we measured mRNA and protein in αTC-1 cells, a glucagon-expressing cell line, treated with rapamycin (30 nM) for 48 hours. These studies showed that inhibiting mTORC1 signaling leads to decreased Kir6.2 and SUR1 protein and mRNA levels (Figure 6, J and K). Overall, our data showed that mTORC1 positively regulates the KIR6.2 and SUR1 protein and mRNA levels (Figure 6, J and K).

Single-cell mRNA expression in α cells from αRaptorKO mice reveals alterations in critical α cell genes. To validate the expression studies and further explore the mechanisms linking mTORC1 to regulation of α cell function and mass, we assessed expression of critical α cell genes using the Fluidigm C1 platform for RNA expression of single pancreatic islet cells (refer to Supplemental Table 3 for the list of genes analyzed). We analyzed an enriched population of α cells by FACS using dispersed islets from 3-week-old αRaptorKO and control mice crossed to Ins1-EGFP and CAG-tdTomato reporter mice, and further sorted these cells based on viability. Single-cell gene analysis validated the decrease in SUR1 and Kir6.2 expression in α cells from young αRaptorKO and further showed reduced Gcg (glucagon) gene expression in these mice (Figure 7A and Table 1). Autophagy-associated genes Ulk1 and Ulk2 were decreased in αRaptorKO, confirming a known role of this pathway in autophagy. Importantly, gene expression of key transcription factors involved in α cell development and maintenance, such as FoxA2, Neurogen3, Gatad4, MafB, Pou3f4, Notch1, Rbpj, and Nkx2.2, were lower in αRaptorKO compared with control α cells (Figure 7A and Table 1). Decreased expression of Ccnb1 (cyclin B) and EIF4E suggests that these genes could be involved in the alteration of α cell proliferation seen in αRaptorKO. Single-cell gene analysis also identified targets involved in exocytosis and glucagon secretion, such as SNAP25 (synaptosomal-associated protein 25), Cacna1s (L-type voltage-dependent calcium channel) and Chrm3 (muscarinic acetylcholine M3 receptor). Endoplasmic reticulum stress–associated genes, Xbp1 and Hsp90ab1, were also decreased in αRaptorKO. FoxA2, Nkx2.2, and Pou3f4 expression was also decreased in αTC-1 cells treated with rapamycin (30 nM) for 48 hours, validating the results obtained by the single-cell analysis (Figure 7B).

The reduction in FoxA2 expression in α cells from αRaptorKO was particularly interesting, as FoxA2 has been shown to directly promote Gcg, SUR1, and Kir6.2 gene transcription in α cells (25–29). Therefore, we hypothesized that mTORC1 positively modulates glucagon and KATP channel expression through regulation of FoxA2 expression. Flow cytometric analysis confirmed that FoxA2 protein levels were also reduced in α cells from young αRaptorKO mice (Figure 7, C and D). In addition, nuclear FoxA2 levels were decreased, as measured by the ratio of signal intensity of nuclear FoxA2 over 4′,6-diamidino-2-phenylindole (DAPI) in the α cells from young control and αRaptorKO mice (Figure 7, E and F).

Discussion

The current studies extend previous reports by uncovering potentially novel insights into the regulation of glucagon secretion and α cell mass by mTORC1 signaling using mice with tissue-specific deletion of Raptor in α cells. This work demonstrates that mTORC1 signaling positively regulates α cell–mass maintenance and glucagon secretion during fasting, hypoglycemia, and glucoprivic signals. Our data show that mTORC1 is dispensable for α cell development, but plays a role in the maintenance of α cells after weaning. Importantly, these experiments uncover a potentially novel role of mTORC1 signaling in the regulation of glucagon secretion by transcriptional regulation of KATP channel subunit expression. We also present a potentially novel role of mTORC1 in controlling critical transcription factors in α cells and identified FoxA2 as a potentially novel mTORC1 target. More importantly, the decrease in glucagon secretion and glucagon content by lack of mTORC1 activity was recapitulated by administration of the clinically used immunosuppressant and mTORC1 inhibitor, rapamycin. Rapalogs (rapamycin analogs including everolimus) are FDA-approved drugs that suppress mTORC1 activity and are routinely used as immunosuppressants in transplantation and for the treatment of several malignancies including insulinomas. Our data suggest that these mechanisms could negatively regulate glucagon levels in vivo and thus inhibit glucagon responses to hypoglycemia in patients taking these medications.

Morphologic studies showed that the decrease in glucagon levels observed in αRaptorKO mice resulted from a loss of α cells after weaning (Figure 3, B–D). Interestingly, no changes in β cell mass were observed in αRaptorKO mice, suggesting that a cell loss in αRaptorKO mice has minor contributions to β cell maintenance during normal conditions. The α cell dynamics associated with loss of mTORC1 signaling are interesting and suggest that mTORC1 is dispensable for α cell developmental programs, but play critical roles during the suckling–weaning transition state.
characterized by a nutritional shift from a fat-enriched maternal milk nutrition to a carbohydrate-rich diet. These results also suggest that mTORC1 in α cells might be important for nutrient-induced signals induced by a carbohydrate-rich diet after weaning. These results are consistent with evidence demonstrating that the weaning period plays a critical role in β cell dynamics and function (30). Our morphological studies and analysis by flow cytometry during the first month of life suggest that mTORC1 activity could have an impact on controlling α cell mass (Figure 3G). In summary, these studies showed that α cell dynamics are altered in αRaptorKO mice during the first month of life and suggest that mTORC1 is important for the transition from a developmental to a mature program in α cells and the nutrition shift associated with weaning.

The current studies show that the physiological role of α cell mTORC1 in the regulation of glucose homeostasis appears to be minor. In particular, hypoglucagonemia failed to decrease glucose levels after 12-hour fasting in αRaptorKO mice (Figure 2D). Similar abnormalities in adaptation to fasting have been reported in other mouse models of near-total α cell ablation and low circulating glucagon levels (18, 31). These results are in marked contrast with the reduction in fasting glucose in mice with pharmacological or genetic inhibition of the glucagon receptor signaling (Gcgr-null), suggesting that low levels of glucagon in αRaptorKO mice are sufficient to mediate proper glucagon receptor signaling and maintain normoglycemia in the fasting state (15, 32). Interestingly, fasting blood glucose was higher in αRaptorKO mice than in control mice after 12 hours of fasting (Figure 2D). Higher glucose production, as shown by a reduced pyruvate tolerance test, could contrib-

Figure 5. Rapamycin treatment in vivo inhibits glucagon secretory responses to hypoglycemia and reduces glucagon content. (A) Experimental design used for administration of rapamycin to 2-month-old wild-type (WT) male mice. Intraperitoneal administration of rapamycin (2 g/kg) or vehicle control was performed every other day for 9 days (arrows). ITT, insulin tolerance test. (B) Body weight and (C) fed glucose levels before rapamycin treatment and day 8 of rapamycin treatment of WT treated with vehicle and WT treated with rapamycin (n = 4–5). (D) Glucose levels after intraperitoneal injection of insulin (1 U/kg) in WT treated with vehicle and WT treated with rapamycin for 8 days (n = 4–5). (E) Glucagon levels at 0 and 30 minutes after insulin injection in the same group of mice (n = 4–5). (F) Glucagon content in isolated islets from WT treated with vehicle and WT treated with rapamycin for 9 days (n = 4; presented as fold change from WT+Vehicle). (G) Glucagon secretion and (H) islet glucagon content from isolated WT islets treated ex vivo with (30 nM) or without rapamycin (n = 3–4 mice/treatment). Data for B and C are shown as means ± SEM. *P ≤ 0.05 (1-way ANOVA with Dunnett’s post-test). Data for D–H are shown as means ± SEM. *P ≤ 0.05 (Student’s 2-tailed t test).
Figure 6. mTORC1 regulates glucagon secretion by alterations in K<sub>K<sub>ATP</sub></sub> channel expression and activity.

Glucagon response from isolated islets to (A) KCl (30 mM) (n = 8 mice) and (B) arginine (ARG, 20 mM) (n = 8 mice) under low-glucose (LG, 1 mM) Krebs buffer. Glucagon response in isolated islets to increasing concentrations of (C) tolbutamide (0–100 μM) under low-glucose conditions (n = 3–4 mice), (D) diazoxide (0–100 μM) under high-glucose (6 mM) conditions (n = 5–8 mice), and (E) diazoxide (0–100 μM) under low-glucose conditions (n = 7–11 mice). (F) K<sub>K<sub>ATP</sub></sub> channel activity during washout of intracellular ATP and (G) current amplitude quantification at 180 seconds in α cells from control and αRaptor<sup>Het</sup> mice (n = 30–41 cells from 3–4 mice). (H) RNA expression of SUR1 and Kir6.2 in FACS-enriched α cell population from control and αRaptor<sup>Het</sup> mice (n = 6). (I) Single-cell analysis of SUR1 expression frequency in α cells from 1-month-old control and αRaptor<sup>Het</sup> mice (n = 20–21 cells from 3–4 mice). (J) RNA expression (n = 7–8) and (K) protein levels of SUR1 and Kir6.2 from αTC-1 cells treated with vehicle or rapamycin (30 nM) for 48 hours (n = 7). All secretion assays (A–E) represent results from 2–3 independent experiments. Data are presented as fold change and shown as means ± SEM. *P ≤ 0.05 (Student’s 2-tailed t test).
α cell mass at 2 months (Figure 2E and Figure 3E). Importantly, 2-month-old αRaptorHET mice also showed impaired glucagon secretion induced by insulin-induced hypoglycemia and glucoprivic conditions, suggesting that mTORC1 activity is important for glucagon secretion (Figure 2E and Figure 5, B and D). Ex vivo studies in isolated islets from αRaptorHET mice also support a role for mTORC1 inhibition in glucagon secretion (Figure 6, A–H).

Finally, αRaptorKO and αRaptorHET mice showed reduced glucagon levels in response to fasting at 2 and 8 months of age (Figure 2, E and I). Although the mechanism for this finding is unclear, it is possible that this reflects defective glucagon secretion in response to explaining this finding. In addition, it is possible that there are compensatory increases in counterregulatory mechanisms, as demonstrated by responses to 2DG administration (Figure 4, C and D). In contrast to the lack of glucose abnormalities, glucagon levels in αRaptorKO were decreased in the fed and fasting state (Figure 2, E and I). These changes could be explained by a marked reduction in α cell mass observed in αRaptorKO, although it is possible that concomitant abnormalities in glucagon secretion can contribute (Figure 3, E and G). In contrast, αRaptorHET mice exhibited a reduction in circulating glucagon levels in the fed and fasting state and these changes were associated with normal α cell mass at 2 months (Figure 2E and Figure 3E).

Importantly, 2-month-old αRaptorHET mice also showed impaired glucagon secretion induced by insulin-induced hypoglycemia and glucoprivic conditions, suggesting that mTORC1 activity is important for glucagon secretion (Figure 2E and Figure 5, B and D). Ex vivo studies in isolated islets from αRaptorHET mice also support a role for mTORC1 inhibition in glucagon secretion (Figure 6, A–H). Finally, αRaptorKO and αRaptorHET mice showed reduced glucagon levels in response to fasting at 2 and 8 months of age (Figure 2, E and I). Although the mechanism for this finding is unclear, it is possible that this reflects defective glucagon secretion in response

Figure 7. mTORC1 regulates the expression of genes involved in glucagon synthesis and regulation of glucagon secretion. (A) Heatmap represents mean Ct of differentially expressed genes (P > 0.05) from Fluidigm single-cell analysis of α cells from 3-week-old control and αRaptorKO mice (n = 3–6 mice) and (B) RNA expression of α cell transcription factors in αTC-1 cells treated with vehicle or rapamycin (30 nM) for 48 hours (n = 4–8/group). (C) FoxA2 protein expression and (D) quantification analysis by mean fluorescence intensity (MFI) in glucagon-positive cells from young control and αRaptorKO mice (n = 3–4). (E) Immunofluorescence images representing FoxA2 (red), glucagon (green), and DAPI (blue) staining of isolated and dispersed islets from young control and αRaptorKO mice. Scale bars: 10 μm. (F) Quantification of the ratio of the signal intensity of nuclear FoxA2 over DAPI in dispersed α cells from young control and αRaptorKO mice (n = 4 mice). Data in (A) represent the mean Ct of differentially expressed genes (P > 0.05) in single cells by Student’s 2-tailed t test and MAST analysis. Data in (B), (D), and (F) are presented as fold change from control and shown as means ± SEM. *P ≤ 0.05 (Student’s 2-tailed t test).
to the increase in circulating amino acids during the fasting state. Taken together, these studies are consistent with the concept that mTORC1 is required to maintain glucagon levels during fasting and in response to hypoglycemia. Interestingly, suppression of glucagon secretion in the fed state appears to not be regulated by the insulin/insulin receptor/mTORC1 axis.

The current studies demonstrate that reduction of mTORC1 signaling reduces glucagon secretion. The similar glucagon secretory responses to KCl treatment in controls and αRaptorHET islets suggested that events distal to calcium influx are conserved and the secretory defect occurred prior to cell depolarization (Figure 6A) (21). In contrast, glucagon responses to arginine, glutamine, or tolbutamide were impaired in αRaptorHET (Figure 6, B and C, and Supplemental Figure 5). While tolbutamide and arginine should also depolarize the α cell, KCl likely provided a much stronger depolarization that supports the sustained activation of L-type Ca²⁺ channels, as opposed to the P/Q-type channels that appear to control glucagon secretion under more physiologic conditions (33, 34). Based on these findings, together with impaired responses to low glucose observed in vivo, we hypothesized that the mechanistic defect in the glucagon secretory pathways in αRaptorHET lied at the level of the K<sub>ATP</sub> channel. Therefore, we assessed glucagon secretion to pharmacologic agents that open or close K<sub>ATP</sub> channels in a dose-response manner (Figure 6, C–E) as described previously (23). The dose-response alterations to diazoxide and tolbutamide, K<sub>ATP</sub> channel activity modulators, are consistent with reduced K<sub>ATP</sub> channels and currents in αRaptorHET α cells (Figure 6, F and G). Titration of K<sub>ATP</sub> channel activity can enhance action potential firing to the point at which voltage-gated Na<sup>+</sup>, Ca<sup>2+</sup>, and K<sup>+</sup> channels undergo voltage-dependent inactivation (35–37) and thus suppress glucagon secretion (as seen with 100 μM tolbutamide in the controls). The lower density of K<sub>ATP</sub> currents in the αRaptorHET α cells is therefore consistent with the suppressive effect of tolbutamide on glucagon secretion occurring at low glucose concentrations, and the lack of effect of diazoxide. Interestingly, the increase in glucagon secretion by diazoxide in control islets is opposite to previously published data showing that a similar concentration of diazoxide inhibits, rather than stimulates glucagon secretion (33). The mechanisms for these differences are not completely clear but it is important to note that a fraction of K<sub>ATP</sub> channels in α cells are open even at low glucose (23, 34). Therefore, it is still possible for diazoxide to open more K<sub>ATP</sub> channels at low glucose and have stimulatory rather than suppressive effects, depending on the fraction of open K<sub>ATP</sub> channels at culture medium conditions used with low glucose. Expression studies demonstrated that the decrease in K<sub>ATP</sub> currents in αRaptorHET α cells resulted in part from lower Kir6.2 and SUR1 mRNA expression in αRaptorHET mice, indicating that mTORC1 signaling controls transcription of K<sub>ATP</sub> channel components (Figure 6, F–H). Reductions in SUR1 and Kir6.2 mRNA expression were validated in single α cell expression analysis in αRaptorKO (Figure 6I, Figure 7A, and Table 1) and reductions in SUR1 and Kir6.2 protein and mRNA levels were validated in αTC-1 cells treated with rapamycin (Figure 6, J and K). Glucagon secretion induced by different secretagogues was reduced by acute treatment of wild-type islets with rapamycin, suggesting that mTORC1 could also modulate glucagon secretion by controlling K<sub>ATP</sub> channel activity, although the precise mechanism is unclear (Figure 5, G and H). Finally, decreased expression of several genes involved in secretory machinery (SNAP25, Chrm3, Cacna1s) in αRaptorKO mice indicates that additional mechanisms could also be involved (Figure 7A and Table 1). Taken together, these results uncovered a previously unknown function of mTORC1 signaling in controlling glucagon secretion by modulating K<sub>ATP</sub> channel activity and expression of K<sub>ATP</sub> channel subunits as well as genes of the secretory machinery.

Our data showed that inhibiting mTORC1 signaling in αRaptorKO mice and chronic rapamycin administration in vivo leads to decreased glucagon content and glucagon secretion. Single-cell gene expression analysis further showed that the decreased glucagon content resulted from reduced glucagon (Gcg) gene expression in αRaptorKO and key transcription factors important for α cell development, maintenance, and glucagon synthesis including FoxA2, Neureg3, Gata4, MafB, Pou3f4, and Nkx2.2 (Figure 7A and Table 1) (38–41). The changes in FoxA2 were particularly interesting, as this transcription factor has been shown to play a major role in regulating Gcg, Suri, and Kir6.2 expression (25–29). FoxA2 protein levels and nuclear FoxA2 levels were also significantly reduced in α cells from young αRaptorKO mice (Figure 7, C–F). The decrease in FoxA2 gene transcription in αRaptorKO mice together with published data linking FoxA2 to Gcg, SURI, and Kir6.2 transcription suggest that reduction in FoxA2 could be the link between mTORC1 and Gcg, SURI, and Kir6.2 transcription in αRaptorKO mice. A decrease in the FoxA2 targets MafB, Pou3f4, and Nkx2.2, also supports the concept that FoxA2-dependent transcription was reduced in αRaptorKO mice (26). Overall, our data identified mTORC1 as a potentially novel regulator of FoxA2 and suggest that this transcription factor links nutrient signaling to transcriptional regulation in α cells.

In summary, dysregulation of glucagon secretion plays a major pathogenic role in the development of hyperglycemia in T2D and failure to secrete glucagon in T1D or advanced T2D patients results in recurrent hypoglycemia. These studies provide potentially novel insights into the molecular mechanisms and signaling pathways regulating glucagon secretion and α cell mass. Our findings identify mTORC1 as a major signaling pathway controlling glucagon secretion under states of low glucose and identify a potentially novel mechanistic link between mTORC1/FoxA2 in transcriptional regulation in α cells. These alterations were recapitulated by treatment with the immunosuppressant rapamycin, a known mTORC1 inhibitor used in the clinic. The findings obtained by rapamycin treatment could have major clinical implications in responses to hypoglycemia in posttransplant diabetics and perhaps explain the defects in counterregulation of hypoglycemia in patients after islet transplantation under chronic immunosuppression by rapamycin analogs (42, 43).

**Methods**

**Animals.** Mice were housed in a pathogen-free environment and maintained on 12-hour light/dark cycle at the University of Michigan Brehm Center Animal Facility. The glucagon-Cre mice (gift from George K. Gittes at the University of Pittsburgh, Pittsburgh, Pennsylvania, USA), express Cre recombinase driven by the glucagon promoter (18). These mice were crossed with Raptor<sup>δδ</sup> (gift from Michael N. Hall and Markus A. Ruegg at the University of Basel, Basel, Switzerland) (19).
We generated 3 experimental groups for all described experiments: controls (Raptor<sup>fl/fl</sup>, Raptor<sup>Cre<sup>/+</sup></sup>, and glucagon-Cre), αRaptor<sup>Cre<sup>/+</sup></sup> (glucagon-Cre; Raptor<sup>Cre<sup>/+</sup></sup>), and αRaptor<sup>HET</sup> (glucagon-Cre; Raptor<sup>fl/fl</sup>). All animals were born in expected mendelian ratios and expected lifespans. Reporter transgenic animals CAG-tdTomato, Ins1-EGFP and CAG-YFP were purchased from The Jackson Laboratory. Reporter transgenic mice, GFP-LC3 (RBRC number RBRC00806) were obtained from RIKEN with the permission of the depositor (Noboru Mizushima, The University of Tokyo, Tokyo, Japan) (20). All metabolic (2 or 8 months) and ex vivo islet secretion (2 months) studies were performed with aged-matched male mice. Islet morphometric analysis utilized age-matched cohorts with male and female mice.

**Metabolic studies.** Body weight and random blood glucose were monitored monthly for a total of 4 months. Fed (9 AM) and fasting (12 hours; 9 PM) glucose, insulin, and glucagon levels were evaluated in 2- and 8-month-old males. Blood was obtained from the tail vein and blood glucose was measured with an Accu-Chek blood glucose meter. Glucagon and insulin levels were measured with ELISAs (Mercodia [25 μl assay] and Alpco, respectively). Active GLP-1 levels in plasma and intestinal tissue were measured by STELLUX Chemi Ultrasensitive Active GLP-1 ELISA (7-36) amide (25 μl assay with a sensitivity of < 0.1 pM). Intrapancreal glucose tolerance test (IPGTT) (2 g/kg), insulin tolerance test (ITT) (1 U/kg), and 2DG (150 mg/kg) were performed by intraperitoneal injections of respective agents in 4- to 6-hour-fasted male mice (44). Hepatic glucose production was measured by intraperitoneal injection of pyruvate (2 g/kg) in 16-hour-fasted male mice.

**Hormone content analysis in tissue extracts.** To measure pancreatic glucagon content and intestinal active GLP-1 content, we dissected and measured the weight of the pancreas and total intestine (intestine was cleaned prior to processing). The tissues were homogenized in acid-ethanol (3 ml) and incubated with gentle rotation at 4°C for 72 hours. The tissue homogenate was centrifuged and glucagon or active GLP-1 level was measured in the collected supernatant (R&amp;D Systems Duoset Glucagon Elisa and STELLUX Chemi Ultrasensitive Active GLP-1 ELISA 7-36). The hormone content measure takes into account the tissue weight and the final volume of supernatant collected (hormone concentration multiplied by tissue weight and divided by the volume of supernatant from tissue extract). Liver glycogen content was measured with a Glycogen Assay Kit (Sigma-Aldrich) following the manufacturer’s instructions.

**Preparation and in vitro treatment with agents.** Rapamycin (LC Laboratories) was dissolved in 100% ethanol and stored at -20°C. The stock solution was further diluted in an aqueous solution of 5.2% Tween 80 and 5.2% PEG 400 with a final concentration of ethanol of 2% (45, 46). Wild-type mice were injected with rapamycin (2 mg/kg) every other day for 9 days. ITT was performed on day 8 between injections. Necropsy was performed on day 9 after the last injection.

**Immunofluorescence and cell morphology.** Pancreata were fixed in 3.7% formaldehyde, embedded in paraffin and sectioned (5 μm). Fluorescence images were acquired using a microscope (Leica DM5500B) and a motorized stage using a camera (Leica DFC360FX) (Leica Microsystems). Cell mass was determined in 5 stained sections (5 μm) separated by 200 μm as previously described (47, 48). The area of insulin, glucagon, somatostatin, and pancreatic polypeptide and the area of each section were quantified with Image Pro Software (version 7; Media Cybernetics). The ratio of the 5 hormone-stained areas to the total pancreatic section area for each mouse was averaged and multiplied by the pancreas weight.

Dispersed cell staining was performed by gently dispersing isolated islets with trypsin-EDTA (0.25% trypsin, 1 mM EDTA). The cells were cytographed using a StatSpin Cytofuge 2 (Beckman Coulter) on slides pre-coated with poly-L-lysine. The cells were immediately fixed with 3.7% formaldehyde for 20 minutes and incubated with Tris-buffered saline containing 0.1% Triton for 10 minutes at room temperature. For FoxA2 staining, the cells were additionally permeabilized with cold methanol at 4°C for 10 minutes (after fixation with formaldehyde). Analysis of nuclear FoxA2 and DAPI signal intensity was done on 16-bit images acquired at ×40 magnification. FoxA2 and DAPI levels were determined by measuring pixel intensity using Adobe Photoshop (Adobe Systems). Assumption of cell death was measured by immunofluorescence-based apoptosis TUNEL assay (Millipore) in 3-5 stained sections (5 μm) separated by 200 μm and costained for glucagon and DAPI. Assessment of cell proliferation was measured by staining and counting Ki67/glucagon<sup>+</sup> cells (Sigma-Aldrich) in 3-5 stained sections (5 μm) separated by 200 μm and costained for glucagon and DAPI. Cell size was calculated by immunofluorescent staining of dispersed islets from 1-month-old mice for glucagon and DAPI and measuring the area of glucagon-positive cells using NIH ImageJ software (v1/49d available at http://rsb.info.nih.gov/ij/index.html). Assessment of Cre-mediated recombination was measured by counting the number of glucagon<sup>+</sup>/reporter<sup>+</sup> cells in dispersed islets from Glucagon-Cre<sup>YFP</sup> and Glucagon-Cre<sup>Tomato</sup> mice (2 months old). Assessment of autophagy was assessed in control and αRaptor<sup>Cre<sup>/+</sup></sup> mice bred to GFP-LC3 mice (20). Islets from 1-month-old Control<sup>αRaptor<sup>Cre<sup>/+</sup></sup></sup> and αRaptor<sup>HET</sup> mice were isolated, dispersed, and cytographed on slides and stained for glucagon, GFP, and DAPI. All antibodies used are summarized in Supplemental Table 1.

**Flow cytometry.** Islets were isolated and incubated overnight in RPMI containing 5 mM glucose. The islets were dispersed into a single-cell suspension and fixed with a BD Pharmingen Transcription Factor Phospho Buffer Set (BD Biosciences). The fixed cells were incubated with conjugated antibodies overnight, at 4°C and with gentle rotation. Dead cells were excluded by Ghost Dye Red 780 (Tonbo). Glucagon, insulin, FoxA2, LC3, Ki67, annexin V, and pS6 (Ser240) expression was measured by FACS (a measure of protein expression or posttranslational modification) per glucagon-positive cell using a BD LSR II (BD Biosciences). The size of live glucagon-positive cells was analyzed by forward scatter area (FSC-A) and glucagon MFI. All antibodies used are summarized in Supplemental Table 1.

**Fluorescence-activated cell sorting (FACS).** Control (glucagon-Cre) and αRaptor<sup>HET</sup> mice were crossed to reporter mice Ins1-EGFP and CAG-tdTomato. The islets from 2-month-old Glucagon-Cre<sup>Ins1GFP;tdTomato</sup> and αRaptor<sup>Cre<sup>/+</sup></sup>GFP-LC3<sup>/</sup> mice were isolated, dispersed, and cytographed on slides and stained for glucagon, GFP, and DAPI. All antibodies used are summarized in Supplemental Table 1.
normalized to the number of cells sorted. The β cell content in different islet batches was consistently around 30% among the different FACs experiments (Supplemental Figure 4). Assessment of viability after sorting in young Glucagon-CreInsGFP;F TAMm and αRaptorKO;Ins1-EGFP;F TAMm mice showed similar viability between control and αRaptorKO mice (87%-98% postsorting viability).

K\textsubscript{ATP} channel activity. Islets from Glucagon-CreF TAMm and αRaptorKO;F TAMm mice, expressing a fluorescent reporter in α cells, were dispersed to single cells and plated overnight on 35-mm dishes as described previously (49). Cells were patch-clamped in the whole-cell voltage-clamp configuration in a heated bath at 32°C–35°C using a HEKA EPC10 amplifier and PatchMaster Software (Heka Electronik) and patch pipettes with resistances of 5–6 MΩm after fire polishing. Whole-cell currents of K\textsubscript{ATP} channels were recorded in response to voltage steps going to −60 and −80 mV from a holding potential of −70 mV. Each cell was culled for 3.5 μl in individual tubes with 3.5 μl collected with a pipette adjusted to 1.5 MΩm in Krebs-Ringer (KRBB) medium containing 6 mM glucose and 0.2% BSA for 1 hour. Groups of 15 islets/mouse were placed in 8 mm cell culture inserts (Millicell), preincubated in high-glucose (HG) KRBB (6 mM glucose), warmed to 37°C, and 0.5 μl of BSA for 1 hour. The cells were captured into a small-sized (5–10 μm) μl of dithiothreitol (10 mM; DTT). Following snap freezing on dry ice, islets from dispersed islets obtained from 1-month-old Control tdTomato cells (n = 3) were isolated, dispersed, and sorted based on GFP (β cells), RFP (α cells), and viability to enrich an α cell population. The viability (87%-97%) and concentration (250-300 cells/μl) of cells were measured using a Countess Automated Cell Counter and mixed with C1 Cell Suspension Reagent (Fluidigm) in a 3:2 ratio. The cells were captured into a small-sized (5–10 μm) or middle-sized (10-17 μm) integrated fluidic circuit (IFC) before undergoing cell lysis, reverse transcription, and cDNA amplification in the C1 Single-Cell Auto Prep instrument. Capture sites containing a single cell were identified by careful examination of the IFC using an Olympus CK2 inverted microscope. Quantitative RT-PCR of 96 Delta iGene Assays was performed by BiomarkHD (Fluidigm) using pre-amplified cDNA from single cells, positive (bulk cell), and negative (no template) controls from each IFC capture. Glucagon cells were identified as single cells with detectable levels of Gcg gene expression in the BioMark HD data (Control n = 10, αRaptorKO n = 19 cells). Delta Gene Assays were validated for single-cell gene expression analysis on a BioMark HD using mouse pancreatic total islet mRNA serially diluted over 12 two-fold dilutions (512 pg to 0.25 pg) and 7 replicates.

Quantitative real-time PCR. For mRNA expression of αTC1 and islets, total RNA was extracted using the RNeasy isolation kit (Qiagen).
Gene expression was assessed by quantitative real-time RT-PCR using Power SYBR Green PCR Mix (Applied Biosystems) on a StepOnePlus detection system (Applied Biosystems) with a standard protocol including a melting curve. Relative abundance for each transcript was calculated by a standard curve of cycle thresholds and normalized to 18S (αTC-1) and β-actin (islets). Primers were purchased from IDT, with the exception of Arx1, MafB, and Nkx2.2, which were purchased from Operon. All primer sequences are available in Supplemental Table 2.

Cell culture studies. αTC-1 cells clone 6 were purchased from ATCC Cell Lines and maintained according to the company’s instructions. For rapamycin experiments, the cells were cultured in 12-well plates and incubated in complete media with vehicle control or rapamycin (30 nM) for 48 hours.

Western blotting. αTC-1 cells were collected and lysed in lysis buffer (125 mM Tris, pH 7; 2% SDS, 1 mM DTT) containing phosphatase (Roche Diagnostics) and protease (Sigma-Aldrich) inhibitor cocktails. Cell lysates were boiled for 10 minutes, and electrophoresed in 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. All antibodies used are listed in Supplemental Table 1. Images were acquired using a Western Bright Sirius kit (BioExpress). Band densitometry was performed by measuring pixel intensity using NIH ImageJ software and normalized to actin in the same membrane. Detection of SURI was accomplished using an anti-SURI antibody (gift from Show-Ling Shyng, Oregon Health & Science University, Portland, Oregon, USA). See complete unedited blots in the supplemental material.

Electron microscopy. Islets were isolated and fixed with 2% glutaraldehyde overnight at 4°C, dehydrated, and embedded in Epon by the Image Analysis Laboratory Core (MiCores, University of Michigan). Ultrathin sections were stained with uranyl acetate and lead citrate. Images were recorded digitally using an electron microscope (JEM-1400 Plus).

Statistics. Assessment of the normality of the data by D’Agostino-Pearson (omnibus K2) supported the use of parametric statistical tests. The statistical analysis for comparisons between 2 groups was performed by unpaired (2-tailed) Student’s t test. One-way ANOVA with post-hoc Dunnett’s multiple comparisons test was used for comparisons among 3 or more groups over several time points (GraphPad Prism). P values less than or equal to 0.05 were considered significant. Analysis of Fluidigm single-cell data identified 55 gene assays (Supplemental Table 3) that qualified for statistical analysis by 2-tailed Student’s t test (≥3 data points/group). Principle component analysis (PCA) was performed using gene expression from 55 genes to evaluate whether the groups were separated distinctively. The heatmap represents the mean C(t) of differentially expressed genes (P > 0.05; absolute fold change > 1.5) from 2-tailed Student’s t-test analysis. We applied one method of statistical analysis to the data set using R-based MAST (model-based analysis of single-cell transcriptomics) package (51). The Gcg gene was identified as significantly different between the groups using MAST analysis.

Study approval. All protocols were approved by the University of Michigan and the University of Miami Animal Care and Use Committees and were in accordance with NIH guidelines.

Author contributions
NB designed and performed the experiments, analyzed results and wrote the manuscript. EBM conceived and designed experiments, analyzed results, and wrote the manuscript. NB, XQD, JC, KC, JG, and MBR performed experiments and analyzed results. GKG, MAR, and MNH generated mice. PEM designed and performed K<sub>ATP</sub> channel activity experiments, analyzed results, and contributed to the writing and discussion of the manuscript. DD, ACP, and all other authors contributed to discussion and reviewed/edited the manuscript.

Acknowledgments
The authors wish to acknowledge funding resources for this essential contribution to this work. E.B.M. is mainly supported by a MERIT award from the Veterans Administration. This work was supported in part by Merit Review Award IBX002728A from the U.S. Department of Veterans Affairs Biomedical Laboratory Research and Development Program. Additional funding includes NIH grants R01-DK073716 and DK084236. N.B. was supported by NIH grant T-32-GM007315 and a Rackham Merit Fellowship (University of Michigan). J.C. was supported by NIH grants T-32-GM007315 and HD007505. The work in the A.C.P. group is supported by grants from the Juvenile Diabetes Research Foundation (JDRF) (grants 5-2011-379 and 2-SRA-2016-149), Department of Veterans Affairs (BX000666), the NIH (DK89572, DK104211, DK106755), and the Vanderbilt Diabetes Research and Training Center (DK020593). D.D. was supported by a Vanderbilt Molecular Endocrinology Training Program grant (ST32 DK07563) and a JDRF Postdoctoral Fellowship Award. We acknowledge support from the Morphology and Image Analysis Core, Metabolomics Core and Phenotyping Core from the Michigan Diabetes Research Center (MDRC) (P30 DK020572). We would like to acknowledge Oliver Umland at the Flow Cytometry Core Facility (Diabetes Research Institute; University of Miami) and Lesley De Armas and Li Pan (CFAR; University of Miami; NIH P30AI073961). We thank Charles Burant, Ken Inoki, John Williams, and Lei Yin (University of Michigan) for discussion of the data.

Address correspondence to: Ernesto Bernal-Mizrachi, Department of Internal Medicine, Division of Endocrinology, Diabetes and Metabolism University of Miami, Miller School of Medicine and Miami VA Health Care System, Miami, Florida 33136, USA. Phone: 305.243.5631; Email: ebernalm@med.miami.edu.


