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

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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.

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

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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 (αRaptor−/−), 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 αRaptor−/− mice and in WT animals exposed to chronic rapamycin administration decreased glucagon content and glucagon secretion. In αRaptor−/− 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 glucose 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/P13K/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...
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 Raptorfl/fl mice (αRaptorKO) (18, 19). Deletion of flanked exon 6 exclusively in α cells from αRaptorKO 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 αRaptorKO mice (Figure 1B). To validate the reduction in mTORC1 signaling in α cells from αRaptorKO 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 αRaptorKO 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 αRaptorKO 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 αRaptorKO or αRaptorHET (glucagon-Cre; Raptorfl/+; Kir6.2−/−; SUR1−/−) 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 2D and H). In contrast, 2-month-old αRaptorKO 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 H).
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

Supplemental Figure 2A). αRaptorKO mice showed increased glucose levels after intraperitoneal pyruvate injection, implying that increased gluconeogenesis could play a role in the resistance of αRaptorKO mice to a decrease in blood glucose during fasting (Supplemental Figure 2B). Consistent with this, liver weight and glycogen content decreased, but tended to be higher in αRaptorKO after overnight fasting (Supplemental Figure 2, C and D). The results of these studies suggest that αRaptorKO mice were more resistant to a decrease in glucose during the first 12 hours of fasting by increased gluconeogenic pathways, but these mechanisms were less effective after prolonged fasting (24 hours). Examination of glucose during fasting at 8 months of age showed a similar reduction in fasting glucose among all groups (Figure 2H). Assessment of glucagon in the fed and fasting states showed lower fed and fasting glucagon levels in αRaptorKO mice at 2 and 8 months (Figure 2, E and I). Interestingly, fed and fasted glucagon levels were also reduced in αRaptorHET mice at 2 and 8 months, but not to the level observed in the αRaptorKO mice (Figure 2, E and I). A trend towards increased glucagon levels was observed in 2- and 8-month-old control mice in response to fasting (Figure 2E). Compared with controls, glucagon levels decreased after 12-hour fasting in young and old 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).
Figure 3. mTORC1 signaling is necessary for maintenance of postnatal α cells. (A) Immunofluorescent staining for insulin and glucagon in pancreatic sections from control, αRaptorHET, and αRaptorKO 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) in 1-month-old (n = 4), and (E) 2-month-old control, αRaptorKO, and αRaptorHET mice (n = 4–5). (F) Glucagon content at postnatal day 1 (newborn) (n = 3) and (G) 2-month-old mice. (H) Quantification of glucagon content (n = 3–4) in 8-week-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 αRaptorHET 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).

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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) in 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 αRaptorHET 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).

The 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 αRaptorHET 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 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 α cell size that was confirmed by cell size measurements (Figure 3, K and L). In addition, the number of glucagon granules appeared reduced 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 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 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
Glucagon secretory responses induced by pharmacologic manipulation of K<sub>ATP</sub> channels in αRaptor<sup>HET</sup> islets is abnormal. To uncover the mechanisms responsible for impaired glucagon secretion in αRaptor<sup>HET</sup> 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 and αRaptor<sup>HET</sup> islets (Figure 6A). Next, we examined the glucagon response to arginine, a secretagogue that induces glucagon secretion in part by modulating K<sub>ATP</sub> channels (21). Glucagon secretion induced by arginine was blunted in αRaptor<sup>HET</sup> islets (Figure 6B). Taken together, these data suggested that the defect in glucagon secretion in αRaptor<sup>HET</sup> mice resided in steps prior to cell depolarization and possibly at the level of the K<sub>ATP</sub> channel. We further evaluated glucagon secretion during pharmacological modulation of K<sub>ATP</sub> channel activity with increasing concentrations of tolbutamide (K<sub>ATP</sub> channel antagonist) and diazoxide (K<sub>ATP</sub> channel agonist). Under low-glucose conditions, when a relatively larger fraction of K<sub>ATP</sub> channels are open, diazoxide further induced glucagon secretion at 1 μM followed by a dose-dependent suppression at 10 and 100 μM in control islets (Figure 6E). Under this condition, αRaptor<sup>HET</sup> islets still failed to respond to diazoxide (Figure 6, D and E). Taken together, these studies suggested that K<sub>ATP</sub> channel function is altered in α cells with reduced mTORC1 signaling.

mTORC1 positively regulates glucagon secretion by modulating K<sub>ATP</sub> channel expression. Next, we tested whether the changes in glucagon secretion obtained by pharmacologic manipulation of K<sub>ATP</sub> channels in αRaptor<sup>HET</sup> mice resulted from alterations in K<sub>ATP</sub> channel activity. Whole-cell patch clamp was performed on α cells at low glucose from Glucagon-Cre<sup>tdTomato</sup> and αRaptor<sup>HET</sup>tdTomato cells expressing a fluorescent reporter in α cells. Following establishment of the whole-cell patch clamp, and subsequent washout of intracellular ATP, K<sub>ATP</sub> channel current was significantly reduced in α<sub>2</sub> SUR1<sup>α</sup> cells compared with controls (Figure 6, F and G). To test the hypothesis that these results could be explained by alterations in K<sub>ATP</sub> subunit expression, we measured the expression of K<sub>ATP</sub> channels subunits Kir6.2 and sulfonylurea receptor subunit 1 (SUR1) in α cells. We isolated an enriched population of α cells by FACS using dispersed islets from αRaptor<sup>HET</sup> 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 α cell population from αRaptor<sup>HET</sup> had decreased SUR1 and Kir6.2 mRNA expression (Figure 6H). Supporting these findings, single-cell mRNA expression for SUR1 in α cells also showed that young αRaptor<sup>Alb-tdTomato</sup> mice had fewer α 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 βTC-1 cells, a glucagon-expressing cell line, treated with rapamycin (30 nM) for 48 hours. These studies showed that inhibiting mTORC1 signaling led to decreased Kir6.2 and SUR1 protein and mRNA levels (Figure 6, J and K). Overall, our data showed that mTORC1 positively regulates KATP channel subunit expression in α cells, which could explain in part the decrease in KATP channel current in αRaptorKO mice.

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 InstL-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, Gata4, 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), Cacnals (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 α 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.

### Table 1. Fluidigm single-cell analysis of α cells from 3-week-old control and αRaptorKO mice

| Gene      | Fold Change | P value  |
|-----------|-------------|----------|
| Ulk2      | −12.79      | 0.002431 |
| SNAP25    | −11.28      | 0.002276 |
| FoxA2     | −5.52       | 0.001291 |
| Neurog3   | −5.18       | 0.000242 |
| Abcc8 (SUR1) | −4.66    | 0.018140 |
| Notch1    | −4.46       | 0.001665 |
| Gata4     | −4.09       | 0.000713 |
| XBP1      | −4.04       | 0.048825 |
| Chrm3     | −3.84       | 0.000878 |
| Mafb      | −3.75       | 0.007382 |
| Pou3f4    | −3.71       | 0.016007 |
| Lmatorl1  | −3.56       | 0.00370 |
| Nkx2-2    | −3.14       | 0.001418 |
| Ccnb1     | −3.00       | 0.000003 |
| Caco1s    | −2.92       | 0.030182 |
| Eif4e     | −2.88       | 0.000655 |
| Rbpj      | −2.65       | 0.017525 |
| Ulk1      | −2.50       | 0.000574 |
| Nkapl (6n2) | −2.29   | 0.016501 |
| Hsp90ab1  | −1.76       | 0.027254 |
| Gcg       | −57.57      | 0.00473 |

Fluidigm single-cell analysis of α cells from 3-week-old control and αRaptorKO mice (n = 3–6 mice). Fold change and P value of differentially expressed genes identified in single cells (Student’s 2-tailed t test and *MAST* analysis).
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 α cell loss in αRaptorKO mice after weaning resulted from decreased proliferation and enhanced apoptosis (Supplemental Figure 4, A–D). The role of autophagy in the α cell loss observed in αRaptorKO mice is unclear and future studies using genetic models or inhibitors of autophagy could be designed to answer this question. In contrast to αRaptorKO mice, αRaptorHET mice also showed reduction in α cell mass only at 8 months, suggesting that long-term reduction of 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 gluconeogenesis, as shown by intraperitoneal pyruvate tolerance test, could contrib-
Figure 6. mTORC1 regulates glucagon secretion by alterations in K_{ATP} 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) \(K_{ATP}\) channel activity during washout of intracellular ATP and (G) current amplitude quantification at 180 seconds in \(\alpha\) cells from control and \(\alpha\)RaptorHET mice \((n = 30–41\) cells from 3–4 mice). (H) RNA expression of \(SUR1\) and \(Kir6.2\) in FACS-enriched \(\alpha\) cell population from control and \(\alpha\)RaptorHET mice \((n = 6)\). (I) Single-cell analysis of \(SUR1\) expression frequency in \(\alpha\) cells from 1-month-old control and \(\alpha\)RaptorKO 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 \(\alpha\)TC-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. *\(P \leq 0.05\) (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
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 the 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_ATP channel. Therefore, we assessed glucagon secretion to pharmacologic agents that open or close K_ATP channels in a dose-response manner (Figure 6, C–E) as described previously (23). The dose-response alterations to diazoxide and tolbutamide, K_ATP channel activity modulators, are consistent with reduced K_ATP channels and currents in αRaptorHET α cells (Figure 6, F and G). Titration of K_ATP channel activity can enhance action potential firing to the point at which voltage-gated Na⁺, Ca²⁺, and K⁺ 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_ATP channels 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_ATP channels in α cells are open even at low glucose (23, 34). Therefore, it is still possible for diazoxide to open more K_ATP channels at low glucose and have stimulatory rather than suppressive effects, depending on the fraction of open K_ATP channels at culture medium conditions used with low glucose. Expression studies demonstrated that the decrease in K_ATP currents in αRaptorHET α cells resulted in part from lower Kir6.2 and SURI mRNA expression in αRaptorHET mice, indicating that mTORC1 signaling controls transcription of K_ATP channel components (Figure 6, F–H). Reductions in SURI and Kir6.2 mRNA expression were validated in single α cell expression analysis in αRaptorHKO (Figure 6I, Figure 7A, and Table 1) and reductions in SURI 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_ATP 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 αRaptorHKO 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_ATP channel activity and expression of K_ATP channel subunits as well as genes of the secretory machinery.

Our data showed that inhibiting mTORC1 signaling in αRaptorHKO 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 αRaptorHKO and key transcription factors important for α cell development, maintenance, and glucagon synthesis including FoxA2, Neog3, 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 αRaptorHKO mice (Figure 7, C–F). The decrease in FoxA2 gene transcription in αRaptorHKO 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 αRaptorHKO mice. A decrease in the FoxA2 targets MafB, Pou3f4, and Nkx2.2, also supports the concept that FoxA2-dependent transcription was reduced in αRaptorHKO 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 recombine driven by the glucagon promoter (18). These mice were crossed with RaportKO (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 \((\text{Raptor}^{\text{HET}}, \text{Raptor}^{\text{Het}}), \text{and glucagon-Cre})\), \(\text{Raptor}^{\text{Het}}\) (glucagon-Cre; \text{Raptor}^{\text{Het}}),\) and \(\text{Raptor}^{\text{Het}}\) (glucagon-Cre; \text{Raptor}^{\text{Het}}).\) 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 \((\text{RBR number RBRCC00806})\) were obtained from RIKEN with the permission of the depositor \((\text{Noboru Mizushima, The University of Tokyo, Tokyo, Japan})\). 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 \((\text{Merckodia}[25 \mu l \text{ assay}] \text{ and Alpco, respectively})\). Active GLP-1 levels in plasma and intestinal tissue were measured by STELLUX Chemi Ultrasensitive Active GLP-1 ELISA \((7-36) \alpha m i d e (25 \mu l \text{ assay with a sensitivity }<0.1 \mathrm{pM})\). Intraperitoneal glucose tolerance test \((\text{IPGTT}) \) \((2 g/kg)\), insulin tolerance test \((\text{ITT}) \) \((1 \text{ U/kg})\), and 2DG \((150 \text{ mg/kg})\) 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 \((\text{intestine was cleaned prior to processing})\). The tissues were homogenized in acid-ethanol \((3 \text{ ml})\) and incubated with gentle rotation at \(4^\circ C\) for 72 hours. The tissue homogenate was centrifuged and glucagon or active GLP-1 level was measured in the collected supernatant \((\text{R&D Systems})\). Duoset Glucagon Elisa and STELLUX Chemi Ultrasensitive Active GLP-1 ELISA \((7-36)\) amide \((25 \mu l \text{ assay with a sensitivity }<0.1 \mathrm{pM})\). Intraperitoneal glucose tolerance test \((\text{IPGTT}) \) \((2 g/kg)\), insulin tolerance test \((\text{ITT}) \) \((1 \text{ U/kg})\) and 2DG \((150 \text{ mg/kg})\) 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.

**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 \((\text{BD Biosciences})\). The fixed cells were incubated with conjugated antibodies overnight, at \(4^\circ C\) and with gentle rotation. Dead cells were excluded by Ghost Dye Red 780 \((\text{Tonbo})\). Glucagon, insulin, FoxA2, LC3, Ki67, annexin V, and pS6 \((\text{Ser240})\) expression was analyzed by MFI \((\text{a measure of protein expression or posttranslational modification})\) per glucagon-positive cell using a BD LSR II \((\text{BD Biosciences})\). The size of live glucagon-positive cells was analyzed by forward scatter area \((\text{FSC-A})\) and glucagon MFI. All antibodies used are summarized in Supplemental Table 1.

**Fluorescence-activated cell sorting (FACS).** Control \((\text{glucagon-Cre})\) and \(\text{Raptor}^{\text{Het}}\) mice were crossed to reporter mice \((\text{Ins1-EGFP})\) and \(\text{CAG-tdTomato})\). The islets from 2-month-old Glucagon-Cre\(\text{Ins1-EGFP}\)\(\text{Raptor}^{\text{Het}}\)\(\text{tdTomato}\) and \(\text{Raptor}^{\text{Het}}\)\(\text{tdTomato}\) mice were isolated, dispersed, and cytofuged 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-Cre\textsuperscript{Ins1GFP;tdTomato} and αRaptor\textsuperscript{Akt1-EGFP;tdTomato} mice showed similar viability between control and αRaptor\textsuperscript{KO} mice (87%-98% postsorting viability).

\( \mathbf{K_{\text{ATP}}} \) channel activity. Islets from Glucagon-Cre\textsuperscript{Ins1GFP;tdTomato} and αRaptor\textsuperscript{Akt1-EGFP;tdTomato} 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 \( \mathbf{K_{\text{ATP}}} \) channels were recorded in response to voltage steps going to −60 and −80 mV from a holding potential of −70 mV. For the \( \mathbf{K_{\text{ATP}}} \) current measurement, the bath solution contained 138 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl\(_2\), 2.6 mM CaCl\(_2\), 5 mM HEPES, and 1 mM glucose (pH 7.4). The pipette solution for these experiments contained 125 mM KCl, 30 mM KOH, 1 mM MgCl\(_2\), 10 mM EGTA, 5 mM HEPES, 0.3 mM Mg-ATP, and 0.3 mM K-ADP (pH 7.15). Following the experiments, α cells were positively identified by immunostaining for glucagon (guinea pig anti-glucagon, 1:5,000; Linco), and appropriate secondary antibody (Alexa Fluor 594 goat anti-guinea pig, 1:200; Invitrogen). Data were analyzed using FitMaster (Heka Electronik).

Islet studies. Islet isolation was accomplished by collagenase digestion as described previously (47). Glucagon secretion ex vivo was assessed by static incubation using isolated islets. Briefly, after overnight culture in RPMI containing 5 mM glucose, islets were preincubated 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-μm cell culture inserts (Millicell), preincubated in high-glucose (HG) KRBB (6 mM glucose) for 1–2 hours and incubated subsequently for 1 hour in each of the following conditions: low-glucose (LG) KRBB (1 mM glucose, with a brief wash with LG between HG KRBB and LG KRBB) and LG KRBB plus arginine (20 mM) or KCl (30 mM). Dose-response experiments measuring glucagon response to diazoxide and tolbutamide were performed using a 30-minute exposure per condition. Glucagon response after acute rapamycin treatment was performed in wild-type islets incubated with 30 nM rapamycin for 30 minutes under increasing glucose concentrations from 1 mM to 24 mM for a total duration of 2 hours under rapamycin treatment. Glucagon response to glutamine and rapamycin (30 nM) was performed after a 30-minute incubation in KRBB containing 4 mM glucose and increasing glutamine concentrations (1, 3, and 5 mM) every 30 minutes for a total duration of 2 hours under rapamycin treatment. Assessment of glucagon content in islets was performed by acid–ethanol extraction using 15 islets per condition. All assays represent results from 2–3 independent experiments. Secreted glucagon levels and islet glucagon content were measured using Glucagon DuoSet ELISA. All glucagon secretion data were normalized to the islet glucagon content and presented as fold change.

Single-cell RT-PCR. The following methods were adapted and modified from Liss et al. and Shiota et al. (21, 50). Red-fluorescent α cells from dispersed islets obtained from 1-month-old Control\textsuperscript{Ins1GFP;tdTomato} and αRaptor\textsuperscript{Akt1-EGFP;tdTomato} were individually hand-picked under an inverted phase-contrast microscope (Leica DMI 3000B). Each cell was collected with a pipette adjusted to 1.5 μl in individual tubes with 3.5 μl water and 5 μl of a mix containing 1 μl 10× RT Buffer (Applied Biosystems), 0.5 μl RNase inhibitor (10 Units; Applied Biosystems), and 0.5 μl dithiothreitol (10 mM; DTT). Following snap freezing on dry ice, cell lysates were subjected to first-strand cDNA synthesis by using a High-Capacity Reverse Transcription cDNA Kit following the manufacturer’s directions (Applied Biosystems). Each cell was treated as separate reaction yielding a final volume of 20 μl. Reverse transcription (RT) reactions were carried out for 60 minutes at 42°C, followed by incubation at 75°C for 15 minutes. All cells underwent quantitative PCR amplification for glucagon and 18S. Only confirmed glucagon-positive cells were included in the analysis.

Single-cell nested RT-PCR for SURI and raptor. For SURI, cDNA from single cells obtained as described above was subjected to a first round of RT-PCR using 8 μl of RT reaction, outside SURI primers (0.5 μM), MgCl\(_2\) (2 mM), dNTPs (0.4 mM), and 2 Units Platinum Taq (Invitrogen), in a total volume of 20 μl. The first RT-PCR was performed under the following conditions: 3 minutes at 94°C, 35 cycles (94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 3 minutes), and final elongation at 72°C for 7 minutes using a MasterCycler pro S (Eppendorf). The product of the reaction was used as template for a second round of PCR using 1 μl of first-PCR product template and 19 μl of a mix containing Power SYBR Green PCR Mix (Applied Biosystems), inside primers and water, and was performed using StepOnePlus detection system (Applied Biosystems). The final product for SURI was visualized in an ethidium bromide–stained agarose gel (2%) by electrophoresis. The results were analyzed by the presence or absence of a positive band. Positive (whole islets) and negative (water) controls were used in every experiment. Primers were purchased from IDT. All primer sequences are available in Supplemental Table 2. Confirmation of Raptor deletion was performed by nested RT-PCR of exon 6 (flanked exon) using cDNA obtained from single cells as described above. The first round of amplification was performed with outside primers (exon 5 and exon 7; Supplemental Table 2). The product of this reaction was used as a template for a second round of amplification using exon 6–specific primers (Supplemental Table 2). See complete unedited gels in the supplemental material.

Single-cell analysis by Fluidigm. Islets from 3-week-old control (Raptor\textsuperscript{f/f};Ins1GFP;tdTomato) and Ins1-GFP;tdTomato (n = 6) and αRaptor\textsuperscript{KO};Ins1-EGFP;tdTomato (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, αRaptor\textsuperscript{KO} 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 αTC-1 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 (αTNC-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. αTNC-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. αTNC-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 Microscopy & 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 distinctly. The heatmap represents the mean Ct of differentially expressed genes (P > 0.05; absolute fold change > 1.5) from 2-tailed Student’s t-test analysis. We applied a second 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\textsubscript{ATP} 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.

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