Decreased IRS signaling impairs β-cell cycle progression and survival in transgenic mice overexpressing S6K in β-cells

Lynda Elghazi1*, Norman Balcazar1*, Manuel Blandino-Rosano1, Corentin Cras-Ménéur1, Szabolcs Fatrai1, Aaron P Gould1, Maggie M Chi2, Kelle H Moley2 and Ernesto Bernal-Mizrachi1.

1Department of Internal Medicine, Division of Metabolism, Endocrinology and Diabetes, Brehm Center for Diabetes Research, University of Michigan, Ann Arbor, Michigan 48105.
2Department of OB/GYN, Washington University School of Medicine, St Louis, Missouri 63110, USA

Correspondence:
Ernesto Bernal-Mizrachi
Email: ebernal@umich.edu

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

Submitted 8 June 2009 and accepted 25 June 2010.

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.

Copyright American Diabetes Association, Inc., 2010
Objectives: The purpose of this study was to evaluate the role of the S6K arm of mTORC1 signaling in regulation of β-cell mass and function. Additionally, we delineate the importance of in vivo S6K activation in the regulation of insulin signaling and the extent to which alteration of IRS signaling modulates β-cell mass and function.

Research design and Methods: The current experiments describe the phenotype of transgenic mice overexpressing a constitutively active form of S6K under the control of the rat insulin promoter.

Results: Activation of S6K signaling in these mice improves insulin secretion in the absence of changes in β-cell mass. The lack of β-cell mass expansion resulted from decreased G1-S progression and increased apoptosis. This phenotype was associated to increased p16 and p27 and decreased Cdk2 levels. The changes in cell cycle were accompanied by diminished survival signals as a consequence of impaired IRS/Akt signaling.

Conclusions: The current work defines the importance of S6K in regulation of β-cell cycle, cell size, function and survival. These experiments also demonstrate that in vivo down-regulation of IRS signaling by TORC1/S6K induces β-cell insulin resistance and that this mechanism could explain some of the abnormalities that ultimately result in β-cell failure and diabetes in conditions of nutrient overload.

Pancreatic β-cells expand their function and mass in both physiologic and pathologic states of nutrient excess and increased insulin demand. Failure of β-cells to adequately expand in settings of increased insulin demand results in hyperglycemia and diabetes. The mechanisms involved in β-cell failure in diabetes are not well understood but determining how glucose and fat overload lead to impaired β-cell mass and function is a key component for understanding the natural history of diabetes and to generate pharmacological agents to treat and prevent this disease.

The mTOR signaling pathway integrates growth factors and nutrient signals and is essential for cell growth and proliferation (1; 2). This pathway is negatively regulated by activation of TSC1/2 and AMPK signaling pathways (3-7). mTOR is a part of two distinct complexes: mTORC1 and mTORC2. The mammalian TORC1 is sensitive to rapamycin and regulates protein translation modulation of ribosomal S6 kinase (S6K), eukaryote initiation factor 4E binding protein 1 (4EBP1), and eukaryote Initiation Factor 4E (eIF4E) (8). mTORC1 is composed of Raptor (regulatory associated protein of mTOR), mLst/GβL and proline-rich PKB/Akt substrate 40 kDa (PRAS40). The mTORC2 complex includes Lst8/GβL, Rictor (rapamycin-insensitive companion of mTOR), PRR5 (proline-rich protein 5) and mSIN (stress-activated-protein-kinase-interacting protein 1) (9; 10). The effects of mTORC1 signaling on cell growth, cell size and cell cycle progression are mediated at least in part by phosphorylation of the downstream effectors S6K and 4EBP1 (11). Activation of S6K by mTOR phosphorylates the ribosomal protein S6 (rpS6). The importance of S6K signaling in β-cells has been assessed in genetically modified models. Global S6K1 knockouts or mice with knock-in at all five phosphorylatable serine residues of rpS6 exhibit decreased β-cell mass, impaired
S6K activation in β-cells

Moreover, S6K is important for insulinoma formation induced by activation of Akt signaling (14). A major limitation for understanding the role of S6K signaling in β-cells using S6K deficient mice is the concomitant alteration in insulin sensitivity by negative feedback on IRS proteins (15-17). In contrast, activation of mTORC1 signaling by conditional deletion of TSC2 in β-cells enhances β-cell mass as a result of increased proliferation and cell size (18; 19). These experiments suggest that mTORC1/S6K signaling is an important regulator of β-cell mass, although the molecular mechanisms and downstream signaling pathways are not well characterized.

Growing evidence suggests that not only fat consumption but also protein intake and an increase in plasma amino acid concentration contribute to the development of glucose intolerance, insulin resistance and type 2 diabetes (20; 21). Recent findings demonstrate that S6K activation in states of nutrient overload modulates insulin sensitivity under conditions of nutrient overload (15-17; 22). In addition, the 4EBP/eIF4E signaling pathway regulates glucose metabolism by modulation of sensitivity to diet induced obesity and insulin resistance (23). While this evidence underscores the importance for mTOR/S6K activation in peripheral tissues as a central player in insulin resistance in nutrient overload, the consequences of activation of this pathway in pancreatic β-cells and the implication of the negative feedback inhibition on IRS signaling in vivo are unknown.

To study the role of S6K activation in β-cells, we developed transgenic mice overexpressing a constitutively active form of S6K in β-cells (S6KCA RIP). S6KCA RIP mice exhibited improved glucose tolerance due to an increase in insulin secretion and without changes in β-cell mass. The lack of β-cell expansion was characterized by a failure of β-cells to progress normally through the cell cycle and increased apoptosis. Interestingly, these alterations resulted at least in part by feedback inhibition on IRS1/2/Akt signaling and increased levels of p16 and p27. The current work defines the importance of the S6K arm activation of mTORC1 signaling in regulation of β-cell cycle, cell size, function and survival. These experiments also demonstrate that in vivo down-regulation of IRS signaling by TORC1/S6K reduces growth factor signaling and this mechanism could explain some of the abnormalities that ultimately result in β-cell failure and diabetes in conditions of nutrient overload.

RESULTS

S6KCARIP mice have increased S6 kinase activity in islet β-cells. To increase S6K signaling in β-cells, we inserted a rapamycin-resistant p70S6K Δ2-46/ΔCT104 (T412E) downstream of the rat insulin I promoter (S6KCA RIP) (24). Three viable and fertile lines were obtained and offspring from two founders with similar transgene expression and disturbances in glucose tolerance were studied. The current studies describe the phenotypic characterization of one of these lines. Transgene expression assessed by immunostaining for HA showed cytoplasmic and nuclear staining in the majority of β-cells (~80-90%) from S6KCA RIP mice (Figure 1A). Immunoblotting for HA also displayed expression of the transgene exclusively in S6KCA RIP (Figure 1B). Transgene expression was 4.6 fold higher than that of endogenous S6K (p<0.05). S6K activity measured by phosphorylation of rpS6 was increased in S6KCA RIP mice (Figure 1C).

S6KCA RIP mice exhibit improved glucose tolerance and enhanced insulin secretion. Glucose levels in 6-hour fasted S6KCA RIP were lower than those of WT mice (Figure 2A). S6KCA RIP exhibited higher insulin values than WT after 6-hour fasting (Figure 2A). No
S6K activation in β-cells

difference in glucose was observed after overnight fasting (time 0; Figure 2B, 2C and data not shown). Glucose tolerance test in 4-month-old S6KCA\textsuperscript{RIP} mice showed lower glucose levels at 30 and 60 minutes (Figure 2B). Improved glucose tolerance was observed in 4- and 18-month old S6KCA\textsuperscript{RIP} mice (data not shown and Figure 2C) indicating that these mice were protected from the impaired glucose tolerance associated with aging (Figure 2C). No changes in insulin sensitivity were observed by insulin tolerance tests (data not shown).

Glucose stimulated insulin secretion demonstrated comparable insulin values after overnight fasting in 4- and 18-month-old S6KCA\textsuperscript{RIP} and WT mice (Figure 2D and E). Insulin levels at 15 minutes after glucose injection were increased in four- and eighteen-month-old S6KCA\textsuperscript{RIP} (Figure 2D and E). No difference between S6KCA\textsuperscript{RIP} and controls were observed after 30 minutes of glucose injection (Figure 2D and E). Static incubation of isolated islets demonstrated increased insulin levels in islets from S6KCA\textsuperscript{RIP} mice when exposed to 2 mM glucose (Figure 2F). Insulin secretion was comparable after stimulation with 20 mM glucose (Figure 2F). Measurements of ATP/ADP content in isolated islets showed that islets from S6KCA\textsuperscript{RIP} mice exhibited higher levels of ATP/ADP in 2 mM glucose (Figure 2G). Similar ATP/ADP levels between WT and S6KCA\textsuperscript{RIP} were obtained with 20 mM glucose.

**Islet morphometry and analysis of cell size.** Immunofluorescence staining for insulin and non-β-cells showed that islet architecture was conserved in S6KCA\textsuperscript{RIP} mice (Figure 3A). Similar β-cell mass between S6KCA\textsuperscript{RIP} and WT mice was comparable at 6 and 18 months of age (Figure 3B and 3C). Islet size distribution between S6KCA\textsuperscript{RIP} and WT was unchanged (data not shown). Double staining for HA and insulin in dispersed islets from S6KCA\textsuperscript{RIP} and WT mice showed that β-cells from transgenic mice appeared to have increase in size (Supplemental Figure 1 in the online appendix available at http://diabetes.diabetesjournals.org). Cell size measurements in pancreatic sections stained for Glut2 and HA demonstrated a 50% increase in the size of β-cells expressing the transgene (Figure 4A and B, p<0.05). The number of β-cells per islets was reduced in S6KCA\textsuperscript{RIP} mice suggesting that islets from these mice exhibited less number of β-cells with increased cell size (Figure 4C, p<0.05).**

**Assessment of Proliferation and apoptosis in S6KCARIP and WT mice.** β-cell proliferation assessed by Ki67 staining showed increased proliferative rate in S6KCA\textsuperscript{RIP} mice (Figure 5A). The increased proliferative rate with absence of changes in β-cell mass suggests that β-cells failed to complete the cell cycle or underwent programmed cell death. Since Ki67 is a marker for G1, G2, S and M phases, we used BrdU and pH3 to specifically assess cell cycle progression through the S and M phases respectively. The rate of BrdU in β-cells was similar between S6KCA\textsuperscript{RIP} and WT mice indicating that β-cells were not progressing to the S phase (Figure 5B). No difference in the rate of pH3 in β-cells was also observed between WT and S6KCA\textsuperscript{RIP} mice (data not shown). Assessment of β-cell apoptosis by cleaved-Caspase 3 staining showed a four-fold increase in apoptosis in S6KCA\textsuperscript{RIP} mice (Figure 5C). Similar increased in apoptosis in S6KCA\textsuperscript{RIP} mice was observed by using TUNEL assay (Figure 5D). The results of these experiments suggest that increased S6K activity in β-cells induced entry but delayed completion of the cell cycle. In addition, these mice also exhibited increased apoptosis.

**Alterations in cell cycle progression in S6KCA\textsuperscript{RIP} mice are associated to increased p27 and p16 levels.** Assessment of cell cycle components responsible for G1-S progression demonstrated that protein levels for Cyclin D1, D2, D3, E and Cdk4 were similar in islets
from S6KCA\textsuperscript{RIP} and WT mice (Figure 6A). In contrast, Cdk2 was reduced in islets from S6KCA\textsuperscript{RIP} mice (Figure 6A). p16 a major regulator of G\textsubscript{1}-S progression in β-cells, was increased in islets from S6KCA\textsuperscript{RIP} mice (Figure 6A). Assessment of the Cip/Kip inhibitors p21 and p27 showed that p27 levels were higher in S6KCA\textsuperscript{RIP} mice (Figure 6A). The number of β-cells with nuclear p27 was increased in S6KCA\textsuperscript{RIP} mice (Figure 6B).

**Decreased IRS signaling in β-cells from S6KCARIP transgenics.** To determine whether islets from S6KCA\textsuperscript{RIP} mice exhibited decreased IRS/Akt signaling, we performed immunostaining for phospho-GSK3β. Marked reduction of phospho-GSK3β staining was observed in islets from S6KCA\textsuperscript{RIP} mice (Figure 7A). Levels for IRS1 and IRS2 were decreased by 50% and 40% respectively in islet lysates from S6KCA\textsuperscript{RIP} mice (Figure 7B, p<0.05). Phosphorylation of IRS1 on Ser 307, one of the many S6K phosphorylation sites on IRS1, was increased in S6KCA\textsuperscript{RIP} mice (Figure 7B). The decreased IRS1 and 2 levels were associated with decreased levels of phospho-GSK3β and phospho-FoxO1 in islets from S6KCA\textsuperscript{RIP} mice (Figure 7B). As expected reduced Akt signaling also resulted in increased TSC2 levels in S6KCA\textsuperscript{RIP} mice (Figure 7B). We next assessed the alterations in IRS/Akt signaling after stimulation with IGF1 (Figure 7C). Phosphorylation of Akt at Ser473 was unchanged in S6KCA\textsuperscript{RIP} mice (Figure 7C). S6KCA\textsuperscript{RIP} mice exhibited reduction of Akt phosphorylation at Thr308. The decreased phosphorylation of Akt at Thr308 resulted in reduced Akt activity as demonstrated by phosphorylation of GSK3β (Figure 7C). Interestingly, phosphorylation of 4EBP, a downstream mTORC1 target, was reduced in S6KCA\textsuperscript{RIP} mice. The results of the current experiments demonstrate that activation of S6K in β-cells reduces Akt activity by decreasing the levels of IRS proteins.

**DISCUSSION**

The results of the current studies serve to elucidate the importance of S6K in regulation of β-cell mass and function and provide insight into the molecular mechanisms involved in the adaptation of β-cells to states of nutrient overload and hyperglycemia. Similar to exposure of β-cells to glucose and amino acids, activation of S6K signaling resulted in improved glucose tolerance, insulin secretion and hyperinsulinemia. These findings show for the first time that activation of S6K induces β-cell insulin resistance by feedback inhibition of IRS signaling. Importantly, these studies also show that S6K regulates IRS2 levels, a major determinant for β-cell proliferation and survival. The β-cell insulin resistance induced by this mechanism had major effects on cell cycle regulation by modulation of Cdk2, p27 and p16 levels. Another major effect of insulin resistance in this model was the increased apoptosis by decreased survival signals from Akt. The current work suggests that one of the major consequences of chronic exposure of β-cells to nutrient overload is the development of impaired IRS signaling. These results serve to elucidate some of the abnormalities observed in adaptive responses of β-cells to nutrient excess and tentatively explain some of the mechanisms involved in glucose toxicity. Finally, these data also demonstrate that the negative feedback of S6K on IRS signaling can be a major modulator of β-cell mass and function in vivo.

The current studies showed that activation of S6K signaling in β-cells causes improved glucose tolerance by increase in insulin secretion. The augmented insulin secretion in the presence of normal β-cell mass suggested that S6K modulates glucose induced insulin secretion. Based on the insulin secretion experiments, it is possible that S6K activation regulates early events on insulin secretion and islet perifusion experiments will required to address this. The mechanism involved in this
process is not completely understood but our experiments suggest that modulation of ATP/ADP ratio could play an important role. Similar to the potentiation of insulin secretion by amino acids reported in the literature, the maximal increase in ATP/ADP ratio observed in islets from S6K in low glucose could prime β-cells to respond to a glucose challenge (25). These findings together with impaired glucose tolerance and defective insulin secretion in S6K1 deficient mice or mice with knock-in at serine residues of rpS6 suggest that S6K modulates insulin secretion. The mechanism implicated in defective insulin secretion in these models is unclear but previous evidence suggest that small cell size with reduction in membrane surface have pronounced effects on insulin secretion (26; 27). The relation between cell size and insulin secretion is intriguing and it is reasonable to speculate that increased cell size and membrane surface size could contribute to the augmented insulin secretion observed in S6KCA RIP mice. Further studies are needed to evaluate this hypothesis and to explore a role of S6K in regulation of distal exocytotic events involved in first and second phases of insulin secretion.

In the present work, overexpression of S6K recapitulated the proliferative and the cell size phenotype described in mice overexpressing a constitutively active form of Akt and mice with conditional deletion of TSC2 in β-cells (18; 28; 29). These results suggest that this kinase relates some of the proliferative and growth signals induced by Akt/mTOR. The effect of S6K on β-cell proliferation is also in agreement with recent findings implicating this kinase in insulinoma formation by Akt activation (14). The absence of β-cell mass changes together with the results of Ki67, BrdU and pH3 measurements suggest that more β-cells enter the cell cycle but G1-S progression is delayed by partial inhibition of the Cyclin D/Cdk4 and Cyclin E/Cdk2 complexes. The alteration in G1-S progression could be explained in part by increased levels of p16 and p27 respectively and lower Cdk2 expression. The normal β-cell mass in this model could be explained by a balance between increased entry but slow progression through the cell cycle combined with increased apoptosis and a long half-life of β-cells. The increased p16 levels is intriguing as this cell cycle inhibitor is a biomarker of aging and is elevated in aged β-cells (30). It is possible that continuous activation of S6K reproduces some of the abnormalities observed in aged β-cells. The augmented levels and nuclear localization of p27 observed in S6KCA RIP mice were most likely caused by a negative feedback inhibition in IRS/Akt signaling. The decreased insulin/IRS/Akt signaling could alter p27 levels by several mechanisms including FoxO1 mediated p27 transcription (31; 32) and/or increased in p27 stability by GSK3β (33). Decreased IRS signaling induces nuclear p27 in β-cells from IRS2 deficient mice (34). The importance of p27 in regulation of β-cell cycle in our model resembles the findings obtained in islets from models of diet-induced obesity and suggesting that S6K signaling might be implicated in the β-cell failure observed in this model (34). Based on the current studies and published evidence, p27 levels are likely to be a marker for impaired IRS signaling in β-cells. This mechanism plays a pivotal role in regulating cell cycle progression under normal conditions or in states of β-cell expansion (34). A major difference between the β-cell mass phenotype of βTSC2−/− and S6KCA RIP mice is that β-cells from βTSC2−/− are probably less susceptible to inhibition on IRS signaling, as the mTOR activation in this model is downstream and not dependent on IRS signaling. In addition, the activation of the mTORC2/Akt pathway could activate survival signals.

The alteration of β-cell survival was the other major component observed by augmented S6K signaling in β-cells. The mechanism involved in apoptosis in these mice could be
multifactorial but our results are consistent with a critical role for a negative feedback of S6K on IRS1/IRS2/Akt signaling. The decreased Akt activity was characterized by decreased phosphorylation of Thr308 but not Ser473 suggesting alterations on PDK1 and not mTORC2 activity. Assessment of downstream Akt targets in islets from S6KCA<sup>RIP</sup> mice implied that the inhibition of IRS2/Akt signaling observed in S6KCA<sup>RIP</sup> induced apoptosis by activation of FoxO1 and GSK3β. Interestingly, Pdx1 levels in S6KCA<sup>RIP</sup> mice were unchanged suggesting this is not a major contributor to the increased apoptosis in these mice (data not shown). The inhibition of IRS signaling by S6K mediated phosphorylation and degradation of IRS1 signaling has been demonstrated in vivo and in vitro (15-17). Less is known about a feedback inhibition on IRS2 signaling but recent data in INS1 cells suggest that IGFI signaling down regulates IRS2 protein by activation of mTOR signaling (35). Our results show for the first time that S6K regulates IRS2 levels in vivo and that this mechanism has major implications for regulation of survival and cell cycle progression in β-cells. The increased apoptosis observed in S6KCA<sup>RIP</sup> mice was unexpected, as S6K has been shown to induce pro-survival signals by inhibiting BAD in lymphocytes (36). This discrepancy could be explained by the fact that β-cells are very sensitive to modulation of IRS2 levels as demonstrated in mice deficient for IRS2 (37). The decrease survival in the S6K mice is in contrast to the absence of apoptosis in βTSC2<sup>-/-</sup> mice (18). These could be explained at least in part by the antiapoptotic effects induced by activation of the eIF4E and mTORC2 activation (21; 38; 39). Taken together, these studies suggest that β-cell insulin resistance induced by a negative feedback of S6K on IRS signaling not only affect cell cycle progression but also impair survival signals. However, activation of S6K signaling alone is not sufficient to induce β-cell failure and diabetes.

The results of the current studies suggest that activation of S6K could play a major role in regulation of β-cell cycle progression and function and implies that S6K relates proliferative but not survival pathways induced by Akt signaling or activation of mTORC1 signaling. Another important conclusion from these studies is that activating proliferation is not always associated with increases in mass and that therapeutic strategies should include pharmacological agents that alter proliferation and apoptosis. These studies collectively show the importance of the S6K arm of mTOR signaling in regulating proliferation, cell size, function and survival and extend the knowledge on the mechanism used for β-cells to regulate G1-S transition and mass. Additionally, these studies also provide information about the adaptation of the β-cell to states of nutrient overload.

**Experimental Procedures: Generation of transgenic mice.** The S6K mutant used in these studies contains deletion of amino acids 2-46, truncation of carboxy-terminal 104 amino acids and Threonine to Glutamatic acid mutation at position 412 (p70S6K ΔD2-46/ΔDCT104 (T412E). This mutant confers rapamycin resistance and increased in basal S6K activity in transfected cells (24; 40). In order to achieve overexpression of a constitutively active form of S6K in β-cells, this construct was inserted behind the rat insulin promoter I (S6KCA<sup>RIP</sup>). Three transgenic founders were obtained and backcrossed to C57BL6J mice, and the N2 and N3 generation from two lines with similar phenotype were analyzed. Wild-type (WT) littermate males with comparable mixed background were used as controls for all the experiments. All procedures were performed in accordance with the Washington University’s Animal Studies Committee.
Metabolic studies. Glucose was measured on whole blood using AccuChek II glucometer (Roche Diagnostics). Plasma insulin levels were determined using a Rat insulin ELISA kit (Crystal Chem). Glucose tolerance tests were performed in 12-hour fasted animals by injecting glucose (2 g/Kg) intraperitoneally as described (28). Insulin secretion in vivo was assessed using 3 g/Kg of glucose intraperitoneally in overnight fasted mice.

Islet studies. Islet isolation was accomplished by collagenase digestion as described previously (28). Insulin secretion was assessed by static incubation of isolated islets as described (41). After overnight culture in RPMI containing 5 mM glucose, islets were precultured for an hour in Krebs-Ringer medium containing 2 mM glucose. Groups of ten islets in triplicate were incubated in Krebs-Ringer medium containing 2 mM glucose or 20 mM glucose for 1 hour. For ATP and ADP measurements, islets were isolated and cultured for 24 hours in RPMI containing 5 mM glucose. Thereafter, islets of similar size were handpicked and precultured for 1h in 0.5 mL Krebs Ringer medium without glucose in organ culture dish. Groups of 20 islets were incubated 1h in Krebs Ringer containing 2 mM and 20 mM glucose respectively and rinsed 3 times in Ringer without glucose. Individual islets were hand picked in 1 μL medium were placed on a slide and frozen immediately in 2-Methylbutane pre-chilled with liquid nitrogen. Freeze dried at -35°C overnight under vacuum with less than 0.01 mm of Hg and removed for extraction. Each islet is extracted in 0.5 μL of 0.1 N NaOH under oil, heated at 80°C for 20 min and neutralized with 0.2 μL mixture of 0.2 M HCl and 0.1 M TrisHCl pH6.8. Samples can be stored at -80°C. 0.1 μL aliquot is obtained for ATP and ADP microanalysis respectively (42). Data is presented as mean of ATP/ATP ratio per islet from at least 20 islets per mouse. For the experiments using IGFI, islets were serum deprived for an hour followed by stimulation with IGFI (100 nM) for 15 minutes.

Immunofluorescence staining. Pancreatic tissues were fixed overnight in 3.7% formalin and embedded in paraffin using standard techniques. The following antibodies were used: Guinea pig anti-Insulin (Dako), mouse anti-HA.11 (Covance), rabbit anti-Glucagon (Chemicon), rabbit anti-Pancreatic Polypeptide (Chemicon), rabbit anti-Somatostatin (Dako), rabbit anti-Glut2 (gift from Bernard Thorens), rabbit anti-Ki67 (Vector), rabbit anti-cleaved Caspase-3 (Cell Signaling), rabbit anti-phospho-GSK3α/β (Ser21/9) (Cell Signaling), mouse anti-BrdU (Amersham), and mouse anti-p27Kip1 (BD transduction). TUNEL assays were performed using the ApopTag Plus fluorescein in situ apoptosis detection kit (Chemicon). The fluorescent secondary antibodies were from Jackson Laboratories. DAPI (Vector) was used to counter-stain nuclei.

Western blotting. Protein from islet lysates were run in polyacrylamide gels and transferred to PVDF membranes. Protein band densitometry was determined using the same membrane by pixel intensity using NIH Image J software (v1.42 freely available at http://rsb.info.nih.gov/ij/index.html) (43) and normalized against that of Actin or Tubulin. Antibodies used for immunoblotting are included in Supplemental Table 1.

Islet morphometry. The β-cell mass was calculated by point counting morphometry from 5 insulin stained sections (5 μm) separated by 200 μm using the BQ Classic98 MR software package (BIOQUANT) as described (28). Cell size was determined in immunofluorescence stained sections for Glut2 and HA. Sectional area of individual β-cells from wild type and transgenics mice that were HA positive was measured using NIH Image J software (v1.42) (43). The number of β-cell per islet was calculated by dividing the number of β-cells in at least eighty islets per animal. Proliferation was assessed in insulin-
S6K activation in β-cells

and Ki67-, BrdU- and phospho-Histone-3 stained sections. At least 3000 insulin stained cells were counted for each animal. Apoptotic rates were determined using cleaved Caspase 3 and TUNEL assay (ApopTag Red In Situ Apoptosis Detection Kit, Chemicon) in insulin stained sections. At least two thousand β-cells were counted in a blinded fashion.

Statistical analysis. All values are expressed as mean ± SEM. For all other comparisons, paired Student’s t test was used. Differences were considered statistically significant at p<0.05.

Author contributions: L.E, N.B, M.B-R, C.C-M, S.F, A.G, M.C, K.M. Researched data. L.E, M.B-R, C.C-M. E.B-M. Wrote/Reviewed/Edited manuscript

ACKNOWLEDGEMENTS
The authors would like to acknowledge Joseph Avruch (Massachusetts General Hospital, and Department of Medicine, Harvard Medical School, Boston, USA) for helpful comments and providing the constitutively active S6K construct. We also acknowledge the support of the Radioimmunoassay (RIA), Morphology core from the Washington University Diabetes Research & Training Center (DRTC). We also thank the Morphology Core from Washington University Digestive Diseases Research Core Center (DDRCC) for histology sections. We thank Pedro Herrera (University of Geneva Medical School, Geneva, Switzerland) for providing the RIP-Cre mice. The anti-Glut2 antibody was kindly provided by Bernard Thorens (Université de Lausane, Lausane, Switzerland). This work was supported by National Institute of Health Grant RO1DK073716-01 (to E.B.-M). E.B.-M. is the recipient of a career Development Award from the American Diabetes Association.

REFERENCES
1. Wullschleger S, Loewith R, Hall MN: TOR Signaling in Growth and Metabolism. Cell 124:471-484, 2006
2. Um SH, D’Alessio D, Thomas G: Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell Metab 3:393-402, 2006
3. Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC: Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell 10:151-162, 2002
4. Inoki K, Li Y, Zhu T, Wu J, Guan KL: TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 4:648-657, 2002
5. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D: Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol 5:578-581, 2003
6. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL, Thomas G: Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell 11:1457-1466, 2003
7. Inoki K, Li Y, Xu T, Guan KL: Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev 17:1829-1834, 2003
8. Harris TE, Lawrence JC, Jr.: TOR signaling. Sci STKE 2003:re15, 2003
9. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM: Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 14:1296-1302, 2004
S6K activation in β-cells

10. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN: Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol 6:1122-1128, 2004
11. Hay N, Sonenberg N: Upstream and downstream of mTOR. Genes Dev 18:1926-1945, 2004
12. Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC: Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. Embo J 17:6649-6659, 1998
13. Ruvsinsky I, Sharon N, Lerner T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zipman P, Meyuhas O: Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes Dev 19:2199-2211, 2005
14. Alliouachene S, Tuttle RL, Boumard S, Lapointe T, Berissi S, Germain S, Jaubert F, Tosh D, Birnbaum MJ, Pende M: Constitutively active Akt1 expression in mouse pancreas requires S6 kinase 1 for insulinoma formation. J Clin Invest 118:3629-3638, 2008
15. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegretti PR, Kozma SC, Auwerx J, Thomas G: Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature 431:200-205, 2004
16. Shah OJ, Wang Z, Hunter T: Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. Curr Biol 14:1650-1656, 2004
17. Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, Barnett J, Leslie NR, Cheng S, Shepherd PR, Gout I, Downes CP, Lamb RF: The TSC1-2 tumor suppressor controls insulin-Pi3K signaling via regulation of IRS proteins. J Cell Biol 166:213-223, 2004
18. Rachdi L, Balcazar N, Osorio-Duque F, Elghazi L, Weiss A, Gould A, Chang-Chen KJ, Gambello MJ, Bernal-Mizrachi E: Disruption of Tsc2 in pancreatic β cells induces β cell mass expansion and improved glucose tolerance in a TORC1-dependent manner. Proc Natl Acad Sci U S A 105:9250-9255, 2008
19. Shigeyama Y, Kobayashi T, Kido Y, Hashimoto N, Asahara S, Matsuda T, Takeda A, Inoue T, Shibutani Y, Koyanagi M, Uchida T, Inoue M, Hino O, Kasuga M, Noda T: Biphasic response of pancreatic β-cell mass to ablation of tuberous sclerosis complex 2 in mice. Mol Cell Biol 28:2971-2979, 2008
20. Krebs M: Amino acid-dependent modulation of glucose metabolism in humans. Eur J Clin Invest 35:351-354, 2005
21. Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N: mTOR, translation initiation and cancer. Oncogene 25:6416-6422, 2006
22. Tremblay F, Mareotte A: Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem 276:38052-38060, 2001
23. Le Bacquer O, Petroulakis E, Paglialunga S, Poulin F, Richard D, Cianflone K, Sonenberg N: Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2. J Clin Invest 117:387-396, 2007
24. Weng QP, Kozlowski M, Belham C, Zhang A, Comb MJ, Avruch J: Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. J Biol Chem 273:16621-16629, 1998
25. Newsholme P, Bender K, Kiely A, Brennan L: Amino acid metabolism, insulin secretion and diabetes. Biochem Soc Trans 35:1180-1186, 2007
26. Giordano E, Cirulli V, Bosco D, Rouiller D, Halban P, Meda P: B-cell size influences glucose-stimulated insulin secretion. *Am J Physiol* 265:C358-364, 1993
27. Swenne I, Crace CJ, Jansson L: Intermittent protein-calorie malnutrition in the young rat causes long-term impairment of the insulin secretory response to glucose in vitro. *J Endocrinol* 118:295-302, 1988
28. Bernal-Mizrachi E, Wen W, Stahlhut S, Welling CM, Permutt MA: Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J Clin Invest* 108:1631-1638, 2001
29. Tuttle RL, Gill NS, Pugh W, Lee JP, Koebeltein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ: Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat Med* 7:1133-1137, 2001
30. Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L, Sharpless NE: Ink4a/Arf expression is a biomarker of aging. *J Clin Invest* 114:1299-1307, 2004
31. Medema RH, Kops GJ, Bos JL, Burgering BM: AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* 404:782-787, 2000
32. Nakamura N, Ramaswamy S, Vazquez F, Signoret S, Loda M, Sellers WR: Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol Cell Biol* 20:8969-8982, 2000
33. Surjit M, Lal SK: Glycogen synthase kinase-3 phosphorylates and regulates the stability of p27kip1 protein. *Cell Cycle* 6:580-588, 2007
34. Uchida T, Nakamura T, Hashimoto N, Matsuda T, Kotani K, Sakaue H, Kido Y, Hayashi Y, Nakayama KI, White MF, Kasuga M: Deletion of Cdkn1b ameliorates hyperglycemia by maintaining compensatory hyperinsulinemia in diabetic mice. *Nat Med* 11:175-182, 2005
35. Briaud I, Dickson LM, Lingohr MK, McCuaig JF, Lawrence JC, Rhodes CJ: Insulin receptor substrate-2 proteasomal degradation mediated by a mammalian target of rapamycin (mTOR)-induced negative feedback down-regulates protein kinase B-mediated signaling pathway in beta-cells. *J Biol Chem* 280:2282-2293, 2005
36. Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ: p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. *Proc Natl Acad Sci U S A* 98:9666-9670, 2001
37. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF: Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900-904, 1998
38. Li S, Takasu T, Perlman DM, Peterson MS, Burrichter D, Avdulov S, Bitterman PB, Polunovsky VA: Translation factor elf4E rescues cells from Myc-dependent apoptosis by inhibiting cytochrome c release. *J Biol Chem* 278:3015-3022, 2003
39. Tan A, Bitterman P, Sonenberg N, Peterson M, Polunovsky V: Inhibition of Myc-dependent apoptosis by eukaryotic translation initiation factor 4E requires cyclin D1. *Oncogene* 19:1437-1447, 2000
40. Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG, Gaffney P, Reese CB, MacDougall CN, Harbison D, Ashworth A, Bownes M: 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol* 7:776-789, 1997
41. Bernal-Mizrachi E, Fatrai S, Johnson JD, Ohsugi M, Otani K, Han Z, Polonsky KS, Permutt MA: Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. *J Clin Invest* 114:928-936, 2004
42. Lowry OH, Passonneau JV: *A flexible system of enzymatic analysis*. New York, Academic Press, 1972
43. Girish V, Vijayalakshmi A: Affordable image analysis using NIH Image/ImageJ. *Indian J Cancer* 41:47, 2004

**Figure 1: Assessment of transgene expression and activity in S6KCA\textsuperscript{RIP} and wild-type mice**

(A) Staining for HA-tag (red) and insulin (green) of pancreatic sections from three-month-old wild-type and S6KCA\textsuperscript{RIP}. (B) Immunoblotting for HA-tag in islets lysates from three-month-old wild type and transgenic mice. (C) S6 Kinase activity was assessed by immunoblotting for phospho-S6 protein using islets from wild type and S6KCA\textsuperscript{RIP} animals cultured overnight in 2 mM glucose, 2% serum. Immunoblotting for Actin was used as loading control. The Figures are representative blots of at least three independent experiments in duplicate (*n*≥6). Scale bars represent 25 µm.
Figure 2: \textit{S6KCA}\textsuperscript{RIP} exhibit improved glucose tolerance and augmented glucose-stimulated insulin secretion

(A) Glucose and insulin levels in four month old wild-type (WT) and \textit{S6KCA}\textsuperscript{RIP} male mice after 6-hour fasting ($n=12$). Intraperitoneal glucose tolerance tests were performed in four (B) and eighteen (C) month-old WT and \textit{S6KCA}\textsuperscript{RIP} male mice. \textit{In vivo} insulin secretion after intraperitoneal glucose in WT and \textit{S6KCA}\textsuperscript{RIP} mice at four (D) and eighteen (E) months of age. (F) Insulin secretion in isolated islets assessed by static incubation. (G) Measurement of ATP/ADP ratio in isolated islets. Data is presented as mean of ATP/ATP ratio per islet from at least 20 islets per mouse ($n\geq3$). \textit{In vivo} data are presented as mean ± SE. (*$p<0.05$; $n\geq6$).
Figure 3: Islet morphometry in wild-type and S6KCA<sup>RIP</sup> animals.
(A) Immunofluorescence staining for insulin (green) and non β-cells (red) in pancreatic sections from WT and S6KCA<sup>RIP</sup>. Assessment of β-cell mass in six (B) and eighteen (C) month-old WT and S6KCA<sup>RIP</sup> male mice. Data are presented as mean ± SE. (*p<0.05; n≥3). Scale bars represent 25 μm.
**Figure 4: Assessment of β-cell size in wild-type and S6KCA\textsuperscript{RIP} animals.**

(A) Pancreatic section from WT and S6KCA\textsuperscript{RIP} mice were immunostained for HA (red) and Glut2 (green). (B) Quantitation of β-cells size in islets from WT and S6KCA\textsuperscript{RIP} mice. Cell size in β-cells from S6KCA\textsuperscript{RIP} mice is presented based on expression of the transgene (HA positive). Data includes measurements of at least 250 cells from 4 WT and S6KCA\textsuperscript{RIP}. (C) Number of β-cells per islet was calculated in at least 80 islets from WT and S6KCA\textsuperscript{RIP} mice. Data are presented as mean ± SE. (*p<0.05; n≥3). Scale bars represent 25 µm.
Figure 5: Assessment of β-cell proliferation and apoptosis in wild-type and S6KCA<sup>RIP</sup> animals

(A) Proliferative index in sections stained for Ki67 and insulin (arrows: double positive cells). (B) Proliferative rate on sections stained for BrdU and insulin. Quantitation of apoptosis by immunostaining for cleaved Caspase-3 (C) and TUNEL assay (D) in insulin-stained sections (apoptotic rate). β-cells positive for cleaved Caspase 3 and TUNEL are marked with arrows. Data are presented as mean ± SE. (*p<0.05; n≥3). Scale bars represent 25 µm.
Figure 6: Assessment of cell cycle components in wild-type and S6KCA^{RIP} mice

(A) Immunoblotting for Cyclin/Cdk complexes using islet lysates from WT and S6KCA^{RIP}. Densitometric analysis of protein bands for Cyclin/Cdk complex components in islet lysates from 4 month old WT and S6KCA^{RIP} mice is presented next to each immunoblot. The Figures are representative blots of at least 3 experiments in duplicate. (*p<0.05; n≥6). (B) Quantitation of nuclear p27 in β-cells (arrows) from WT and S6KCA^{RIP} mice (n≥3). At least 2000 β-cells were included in the analysis. Data is presented as mean ± SE. (*p<0.05). Scale bars represent 25 µm.
**Figure 7: Immunoblotting for IRS/Akt signaling components in wild-type and S6KCA\textsuperscript{RIP} mice**

(A) Immunofluorescence staining using anti-phospho-GSK3 (red) and insulin (green) in pancreatic sections from wild-type and S6KCA\textsuperscript{RIP} mice. (B) Immunoblotting and quantitation for different components of IRS/Akt signaling in islet lysates from WT and S6KCA\textsuperscript{RIP} mice. Scanning densitometry of protein bands and quantitation is presented next to the respective immunoblot (n≥4). Immunoblotting for Actin or Tubulin were used as loading control. Quantitation of the data is presented as percentage of the control (n=4). (C) Assessment of phosphorylation of Akt, GSK3β and 4EBP in islet from WT and S6KCA\textsuperscript{RIP} mice after stimulation with IGF1 (100 nM) for 15 minutes. (n≥4). Data is presented as mean ± SE. (*p<0.05). Scale bars represent 25 µm.