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TSC1/TSC2 Signaling in Pancreatic β-Cells

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Abstract: The Tuberous Sclerosis complex (TSC) integrates metabolic and growth signals. Recent data demonstrate that this pathway is a major player in regulation of metabolism and energy balance. In this review, we will focus on the role of TSC in modulation of β-cell mass and function.

Keywords: Tsc1, Tsc2, mTOR, Rheb, nutrients, β-cells.

The capacity of β-cells to adapt to stress conditions is a major factor for the development of diabetes. Type 1 diabetes is characterized by autoimmune destruction of β-cells. In contrast, in physiologic and pathologic states of nutrient excess and increased insulin demand pancreatic β-cells adapt by expanding function and mass. β-cell responses to nutrient excess occur by several mechanisms, including hypertrophy and proliferation of existing β-cells, increased insulin production and secretion, and formation of new β-cells from progenitor cells. Failure of the pancreatic β-cells to adequately adapt and expand in settings of increased insulin demand leads to hyperglycemia and type 2 diabetes. The signals driving the adaptation of β-cells during the different stages of type 2 diabetes are not completely understood, but growth factors including insulin and nutrients are involved. Insulin, as well as other growth factors, glucose and certain amino acids are known to regulate β-cell mass and function. Work form multiple laboratories have identified IRS/PI3-kinase/Akt signaling as a major component linking growth factor insulin and incretins signaling to the regulation of β-cell mass. Downstream of PI3K/Akt, the TSC (Tuberous Sclerosis Complex) is an important component in this process because it integrates signals from both growth factors and nutrients. Recent reports have identified a critical role for the TSC/mammalian target of rapamycin (mTOR) pathway on whole body metabolism. In this review we will focus on the evidence indicating the importance of TSC and downstream events in modulation of β-cell mass and function.

TSC1/TSC2 INTEGRATES GROWTH FACTOR AND NUTRIENT SIGNALS

The tumor suppressor genes, TSC1 and TSC2, are involved in regulation of cell growth [1] and proliferation [2-4]. Although TSC1/TSC2 are present in most eukaryotes they are not present in the yeast *S. cerevisiae,* and *C. elegans*. *TSC1* consists of 21 exons [5] and encodes for Hamartin. The major function of TSC1 is to stabilize TSC2 and prevent its ubiquitin-mediated degradation [6, 7]. *TSC2* has 41 exons and encodes for Tuberin. TSC2 contains the GTPase activating function (GAP activity) of the TSC1-TSC2 complex (Fig. **1**). The two proteins form a ubiquitous cytoplasmic heterodimer called Tuberous sclerosis complex (TSC). This complex acts as a functional inhibitor of mTOR. Studies in human, mice, *Drosophila* and yeast models strongly suggest that their gene products are interdependent and that these proteins function primarily as a complex. Mutations of TSC lead to Tuberous Sclerosis, an inherited autosomal dominant pathology with a high penetrance. The symptoms range from hypo-pigmented skin to epilepsy, severe mental retardation, to renal failure. Mutation of both alleles of TSC1 or TSC2 in affected tissues leads to the development of tumor-like hamartomas in various organs [8]. Deletion of one allele of either gene leads to similar pathology with increased incidence of tumors [9, 10]. Homozygous global deletions of TSC1 or TSC2 are embryonic lethal [10, 11].

mTOR positively regulates anabolic processes that include transcription, protein synthesis (translation or post-translational events), ribosome biogenesis, nutrient transport, and mitochondrial metabolism. On the other hand, TOR negatively regulates catabolic processes such as mRNA degradation, ubiquitin-dependent proteolysis, autophagy, and apoptosis [12]. Besides its functions in the regulation of protein synthesis, cell growth and proliferation, mTOR is also implicated in transcriptional regulation in response to nutrients and stress by controlling the phosphorylation and cellular localization of various transcription factors [13-17]. mTOR protein kinase is found in two functionally and structurally separate complexes: mTOR complex 1 (mTORC1) is rapamycin sensitive and controls cell growth. It is composed of TOR bound to Raptor (regulatory associated protein of mTOR), mLst/GβL and proline-rich PKB/Akt substrate 40 kDa (PRAS40) (Fig. **1**) [18, 19]. On the other hand, mTORC2 (mTOR complex) 2 is not regulated by the TSC1-TSC2 complex and is unaffected by rapamycin except that prolonged exposure to the compound

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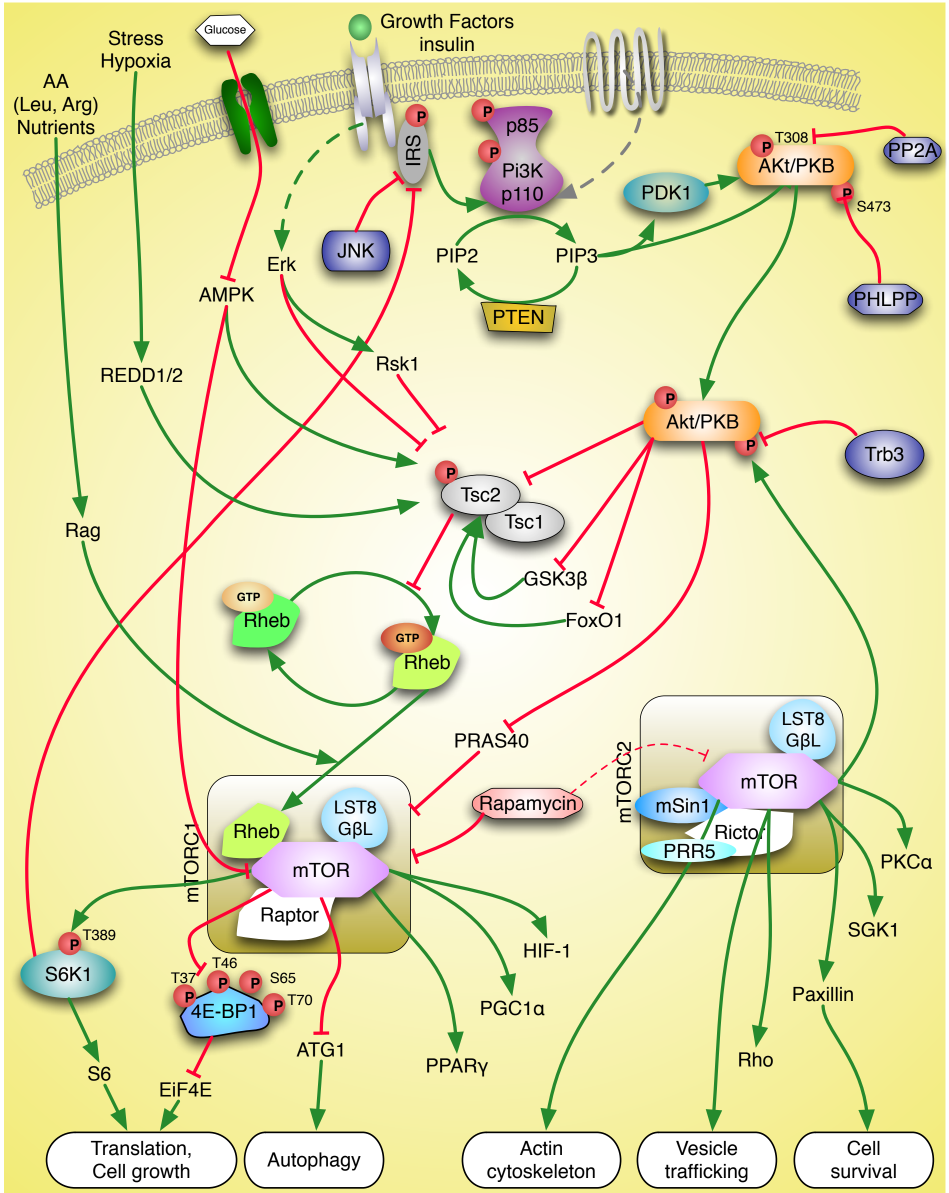

Fig. (1). Schematic diagram of TSC activation and signaling. TSC/mTOR signaling can be activated by insulin, growth factors and nutrients. Activation of insulin and growth factor receptors is mediated by Akt signaling. A critical step leading to activation of Akt is the generation of phosphatidylinositol (3,4,5) P3 phosphate (PIP3) by Phosphoinositide 3-kinase (PI3K). PIP3 binding to the Pleckstrin homology-domain (PH) of Akt/PKB and phosphoinositide-dependent kinase-1 (PDK1) recruits these proteins to the plasma membrane favoring phosphorylation of T308 (309 in AKT2 and 305 in AKT3). Phosphorylation of S473 by the mTORC2 (mTor/Rictor/GβL/mSin/PRR5) complex is necessary for full activation of Akt. The inactivation of Akt/PKB signaling is mediated by protein phosphatase 2A (PP2A) and the α isoform of PH-domain leucine-rich repeat phosphatase (PHLPP)-mediated dephosphorylation of T308 and S473 respectively. Akt/PKB activity is also negatively regulated by the dephosphorylation of PIP3 molecules by the phosphatase and Tensin homologue deleted on chromosome 10 (PTEN). Another level of regulation is achieved by binding to Akt-interacting proteins that lack significant kinase activity like the mammalian homolog of *Drosophila Tribbles* (TRB3) among others. Recent evidence suggests that increased mTORC1 (mTOR/Raptor/GβL) signaling inhibits insulin signaling by phosphorylation of IRS1 and possibly IRS2 in a ribosomal S6 kinase (S6K)-dependent manner. Akt phosphorylates TSC2 and disrupts the complex. The disruption of the complex inhibits the GTPase activating function (GAP activity) of the TSC1-TSC2 complex towards Rheb and favors the GTP bound form of Rheb (active). Active Rheb phosphorylates and activates the rapamycin sensitive mTORC1 (composed of TOR bound to Raptor, mLst/GβL and proline-rich PKB/Akt substrate 40 kDa (PRAS40)). Akt can also activate mTORC1 by phosphorylation and inhibition of PRAS40. Active mTORC1 phosphorylates and activates S6K. In addition, phosphorylation of the complex eIF4E-4E-BP by mTORC1 at multiple sites leads to dissociation of eIF4E from 4E-BP leading to protein synthesis. Additional mTORC1 targets include ATG1, PPARγ, PGC1α and HIF-1. Glucose can indirectly modulate mTORC1 by inhibiting AMPK activity. Amino acid activation of mTORC1 is more complex and includes the Rag GTPases. Rag GTPases are involved in transporting mTORC1 to facilitate the ability of Rheb-GTP to activate mTORC1. Stress conditions such as hypoxia can regulate TSC complex through REDD1/2. Finally, mTORC2 is not regulated by the TSC complex and is involved in multiple biological processes that include actin cytoskeleton remodeling, survival and vesicle trafficking.

may inhibit mTORC2 in a subset of cancer cells [20]. It is composed of TOR linked to Lst8/GβL, Rictor (rapamycin-insensitive companion of mTOR) [21], PRR5 (proline-rich protein 5) and mSIN (stress-activated-protein-kinase-interacting protein 1) (Fig. **1**) [22-25]. All the components of this complex are essential since deletion of any of the partners results in embryonic lethality. This complex regulates Actin cytoskeleton remodeling and certain AGC kinases such as Akt by phosphorylation on Ser^{473} [22] and PKCα [12, 14, 15, 21, 24-27]. Since there is no strong evidence linking TSC to regulation of mTORC2, we will focus our discussion on mTORC1.

UPSTREAM SIGNALS OF TSC1/TSC2/MTORC1

The TSC1/TSC2 complex is often seen as a traditional target of Akt/PKB (reviewed in [26], but a diverse range of additional regulating kinases have since been identified and now over thirty different proteins are known to interact with TSC (reviewed in [28]). Under normal conditions, the TSC1-TSC2 complex serves as a GAP activity towards Ras enriched in brain tissue (Rheb), favoring the GDP-bound form of Rheb (inactive) (Fig. **1**) [29]. Upon stimulation with growth factors or insulin, Akt/PKB will inhibit the TSC complex by phosphorylating TSC2 on four different residues (Ser^{939}, Ser^{1086}, Ser^{1088} and Thr^{1422} [30-32]). The mechanisms responsible for activation of Rheb by Akt are not completely understood but TSC2 phosphorylation by Akt dissociates the TSC1-TSC2 complex resulting in derepression of Rheb and up regulation of mTORC1 [29, 30, 32-37]. Modulation of FoxO1 and GSK3β are two additional downstream Akt regulatory molecules affecting the stability of the TSC complex. Mutation of all the Akt phosphorylation sites on FoxO1 facilitates its binding to TSC2 and stabilizes the TSC complex (Fig. **1**), suggesting that this is an additional mechanism whereby Akt can modulate TSC signaling [38]. GSK3β is one of the main effectors of the Wnt signaling pathway and is also inactivated by Akt. When active, GSK3β phosphorylates and activates the GAP function of TSC2 in an AMPK-dependent manner [39].

Recent studies identified a TSC complex-independent activation of mTORC1 by Akt signaling. This mechanism implicates the PRAS40 protein [19]. In response to growth factors, Akt phosphorylates and inhibits PRAS40, releasing the inhibition on mTORC1.

The insulin and growth factor receptors can also activate the Ras/MAPK pathway independently of IRS. Erk-dependent phosphorylation of TSC2 leads to the dissociation of the TSC1-TSC2 complex and activation of mTOR signaling [40]. Erk also activates p90 Ribosomal S6 Kinase 1 (Rsk1), which in turn phosphorylates TSC2 at Ser^{1798}, further inhibiting the formation of the TSC complex [41]. Additional growth factor-independent pathways can also regulate TSC. AMP-activated protein kinase (AMPK) is an energy sensor and a potent activator of the TSC complex. AMPK can prime TSC2 at Ser^{1345}, allowing subsequent activating phosphorylation by GSK3β [39]. In conditions of low energy, AMPK is activated resulting in activation of the TSC complex and suppression of protein synthesis.

The signaling events relating the effect of amino acids to different biological responses include mTORC1. Amino acids are important regulators of TORC1 activity, although the mechanism responsible for this activation is not as well characterized as the regulation by insulin or growth factors. Rag GTPases have been identified as important regulators of this process by transporting mTORC1 to an ill defined location and facilitating the ability of Rheb-GTP to activate mTORC1 (Fig. **1**) [42, 43]. Ste20-related kinase, MAP4K3 and the class III PI3K mVps34 have also been implicated as mediators of amino acids signaling to mTOR [44-46]. Amino acids have been recognized as potent signaling mediators in pancreatic β-cell function. In addition to their role in regulating insulin and glucagon secretion [47, 48], they also have been implicated in modulation of β-cell proliferation [49]. *In vitro* studies on primary islets have further demonstrated how amino acids modulate β-cell replication [50-53]. Among all amino acids, leucine, in the presence of glutamine, exerts the strongest effect on mTORC1 [54]. The importance of amino acids has also been emphasized in different animal models of malnutrition [55]. Maternal undernutrition during gestation results in fetuses with intrauterine growth retardation that leads to decreased β-cell mass by low proliferation rates and impaired glucose metabolism that persist during adult life [55]. Finally, stress and hypoxia also act as growth and protein synthesis limiting factors through the activation of the regulated in development and DNA damage responses protein 1 (REDD1), REDD1 enhances TSC2 activity leading to inhibition of Rheb. Deletion of REDD1 demonstrated that this protein was key in the regulation of S6 ribosomal kinase (S6K) through TSC under hypoxic conditions, for instance [56, 57].

DOWNSTREAM SIGNALS OF MTORC1

Activation of mTORC1 signaling leads to increased translation, including the synthesis of secreted proteins. mTORC1 constitutes the rapamycin-sensitive arm of mTOR signaling and phosphorylates and modulates the activity of ribosomal S6 kinase 1 and 2 (S6K1 and 2) and eukaryote initiation factor 4E binding protein 1 and 2 (4E-BP), key regulators of protein translation, cell growth and proliferation (Fig. **1**).

REGULATION OF β-CELL MASS AND FUNCTION BY TSC1/TSC2 SIGNALING

TSC2. The direct implication of TSC2 in the regulation of β-cell mass and carbohydrate metabolism *in vivo* was demonstrated through two independent genetic models of conditional deletion of *TSC2* by crossing mice carrying a TSC2 floxed allele with mice in which Cre expression is under the control of the *Insulin* promoter (RIP-Cre) [58, 59]. Mice with a conditional deletion of TSC2 in β-cells displayed decreased glucose levels and hyperinsulinemia in the fasting and fed state. These changes were associated with improved glucose tolerance that was maintained with aging. As a result of the absence of TSC2, the phosphorylation of the downstream targets of mTORC1, S6K, S6 and 4E-BP was significantly increased and β-cells grew both in size [59] and number [58]. In a separate report, Shigeyama *et al.* demonstrated that conditional TSC2 deletion in β-cells exhibited a similar phenotype including lower glucose levels, hyperinsulinemia and improved glucose tolerance during the first 30 weeks of life. These metabolic changes were associated with increased β-cell mass and cell size.

Interestingly, at 40 weeks of age these mice developed progressive hyperglycemia and hypoinsulinemia accompanied by a reduction in β-cell mass. Both of these studies did not evaluate the effect of TSC2 deletion in β-cells during developmental stages. The differences in glucose phenotype between these reports are most likely explained by different genetic backgrounds of the floxed TSC2 mice and the use of different RIP-Cre lines.

TSC1. Most recently, the importance of TSC1 in metabolism and β-cell mass and function has been investigated. Since complete inactivation of TSC1 in mice is embryonic lethal [10, 11, 60], the investigation of the importance of this protein in the pancreas has been studied by conditional deletion of TSC1 in pancreatic β-cells using the *RIP2-Cre* transgenic mice [61, 62]. Given that TSC1 and TSC2 products are interdependent and these proteins function primarily as a complex, it was expected that conditional deletion of TSC1 in β-cells would produce a similar phenotype to that observed with TSC2. Interestingly, these mice exhibited hyperphagia, obesity and insulin resistance that could be explained by the deletion of TSC1 in the hypothalamus due to the ectopic expression of the *Rip2-Cre* transgene [62]. The energy balance phenotype was associated with hyperglycemia and reduced β-cell mass after 12 weeks of age. Examination of the β-cell phenotype in younger mice demonstrated that these mice had lower glucose levels and improved glucose tolerance that was associated with a modest increase in total β-cell area and individual cell size. Although some evidence suggest that mTORC1 is involved in insulin mRNA translation [63], the effect on insulin synthesis by activation of mTOR signaling requires further pulse labeling experiments and detailed assessment of insulin content per cell should be performed to demonstrate this action of mTOR.

Rheb. In addition to deleting the components of the TSC complex, mTOR could also be induced by overexpression of Rheb. This strategy was evaluated by overexpressing Rheb in transgenic mice using the rat insulin promoter [64]. These mice exhibited improved glucose tolerance and lower glucose levels. The glucose-stimulated insulin secretion was increased as a result of elevated β-cell mass. In this model most of the changes in mass appear to be caused by increased cell size. Moreover, Rheb transgenics were more resistant to obesity- and streptozotocin-induced diabetes [64].

mTORC1. The role of mTORC1 in β-cells has been explored by *in vivo* and *in vitro* experiments using rapamycin. Rapamycin treatment of human and rodent islets inhibited ^{3}H Thymidine incorporation and cell cycle progression suggesting that mTORC1 regulates growth and proliferation of β-cells *in vitro* and *in vitro* [49, 53, 65-67]. Rapamycin has also been used *in vivo* in different settings. Rapamycin treatment resulted in reduced β-cell proliferation but not function in a pregnancy model [65]. Administration of rapamycin to *Psammomys obesus*, an animal model of type 2 diabetes, significantly worsened the diabetic phenotype as a result of insulin resistance, reduction of β-cell mass and increased apoptosis [68]. These studies suggest that mTORC1 regulates β-cell mass in adaptation to signals that induce β-cell mass such as insulin resistance. A more direct effect of mTORC1 in β-cells comes from the use of rapamycin in animal models with conditional activation of mTOR signaling in β-cells. Inhibition of mTORC1 by rapamycin treatment decreases the β-cell mass expansion and cell size of mice with deletion of *TSC2* or *TSC1* [58, 64, 69]. Similar findings were observed in transgenic mice overexpressing Rheb treated with rapamycin. These changes were accompanied by reversion of improved glucose tolerance and hyperinsulinemia. While the reversal of the metabolic phenotype in these models could be explained by changes in β-cell mass, it is also possible that inhibition of mTORC1 could alter insulin secretion. It is important to note that the contribution of proliferation to augmented β-cell mass has not been reproduced in all the models of genetic activation of mTORC1 signaling [64]. The explanation for this is unclear but it is possible that there are different downstream targets for TSC1, TSC2 or Rheb. Most recently, inhibition of mTORC1 by rapamycin reduced proliferative responses induced by conditional activation of Akt signaling [70]. More importantly, these studies demonstrated that activation of mTORC1 induces cyclin D2 and D3 levels. The changes in cyclin D2 levels resulted from regulation of cyclin D2 synthesis and stability [71]. A potential limitation of these studies is that different rapamycin protocols have been used and that this agent can induce systemic insulin resistance making it difficult to interpret some of the specific alterations in β-cells. Further experiments using mice with a conditional deletion of raptor will elucidate the role of mTORC1 in β-cell mass and function.

S6K. The ribosomal protein S6 kinase (S6K) is described as a regulator of cell growth, protein translation and proliferation [72]. S6K1 and S6K2 are the products of alternative splicing of a single transcript and both proteins exist in two forms (short and long). Only the short form of S6K1 ($p70^{S6K1}$) is cytoplasmic. The activity of S6K1 is regulated by both mTORC1 and PDK1. Several downstream targets of S6K have been identified. S6K1 and S6K2 regulate the 40S ribosomal protein S6, the elongation initiation factor 4B (eIF4B), SKAR and the elongation factor 2 kinase (eEF2K) [35]. S6K1 and S6K2-mediated phosphorylation is required for full activation and subsequent induction of the 40S ribosomal protein S6. S6 can then induce cell growth and proliferation. S6K also negatively regulates IRS1 and 2, therefore inactivating the PI3K signaling pathway [16, 73-76]. The importance of S6K signaling in β-cells has been assessed in genetically modified models. S6K1-deficient mice are viable and fertile and only present mild phenotypes during development because of a concomitant increase in S6K2 [77, 78]. Mice deficient in S6K1 further display glucose intolerance and hypoinsulinemia with impaired insulin secretion. The pancreatic β-cells displayed a reduced size and decreased insulin transcription. This study demonstrated the importance of S6K1 in regulating glucose homeostasis as well as cell growth. Interestingly, when placed on a special diet, *S6K1*-deficient mice were resistant to age and high fat diet-induced obesity. These animals remained insulin-sensitive due to the loss of the negative feedback loop from S6K on IRS1 and IRS2. These results and studies from other groups suggest an *in vivo* role for S6K in desensitizing tissues to insulin [16, 75, 76]. Although the significance of this feedback regulation on IRS1 has recently been questioned [79]. In contrast, S6K2-deficient mice are phenotypically similar to the control, suggesting that S6K1 might be more important than S6K2 in glucose homeostasis [80]. Recent findings also showed that S6K is important for insulinoma formation induced by activation of Akt signaling, implicating this kinase in regulation of β-cell cell cycle progression [81]. Less is known about the signaling events downstream of S6K. The importance of ribosomal S6 protein was assessed by knock-in mice, whose rpS6 contains alanine substitutions at all five phosphorylatable serine residues [82]. These mice exhibit impaired glucose tolerance, lower insulin levels and increased insulin sensitivity. The pancreatic insulin content was reduced in these mice and this finding was not associated with alterations in β-cell mass implying an effect of S6 protein on insulin synthesis. The similarity of this phenotype with that of S6K deficient mice suggests that ribosomal S6 protein is a critical substrate in relating metabolic signals from S6K.

4E-BP/EIF. The 4E-BP proteins are repressors of the translation initiation factor 4E (eIF4E) and therefore inhibit protein translation. Phosphorylation of the eIF4E-BP complex by mTORC1 at multiple sites leads to dissociation of eIF4E from 4E-BP allowing its binding to eIF4F and eIF4G. This promotes the translational machinery of mRNAs with high 5′-UTR secondary structures, such as those that encode ribosomal proteins, elongation factors and other proteins involved in the assembly and function of the translational machinery [83-85]. The combined disruption of 4E-BP1 and 4E-BP2 in mice increased their sensitivity to diet-induced obesity by accelerated adipogenesis. The animals displayed increased insulin resistance associated with increased ribosomal protein S6 kinase (S6K) activity and impaired IRS2/Akt signaling in peripheral tissues. Unfortunately, the β-cell phenotype of these animals was not analyzed [86]. Most recent experiments have revealed an important role for 4E-BP in protection against endoplasmic reticulum (ER) stress in β-cells. These studies demonstrated that 4E-BP1 expression was increased in islets under ER stress in several mouse models of diabetes [87]. The induction of 4E-BP1 levels resulted from direct transcriptional activation of the *Eif4E-BP1* gene. Most importantly, islets from 4E-BP1 null mutant mice were more susceptible to ER stress-induced apoptosis suggesting that 4E-BP1 could be a survival factor for β-cells.

CONCLUDING REMARKS

The current evidence suggests that the TSC/mTORC1 signaling pathway plays a critical role in regulation of metabolism and energy balance. In particular, this signaling pathway is responsible for relating metabolic and growth signals to modulate β-cell mass and function *in vivo*. Future experiments are needed to determine the specific role of the different components of the pathway in the modulation of cell size, proliferation, energy balance, mitochondrial function and β-cell mass, proliferation and insulin secretion.

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