Investigating the Hippo signaling pathway in pancreatic β-cells

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I Summary

Apoptosis and loss of function are hallmarks of pancreatic β-cell failure in both type 1 and type 2 diabetes. Targeting β-cell apoptosis and dysfunction therefore represents an attractive therapeutic approach to the treatment of both T1D and T2D. The initial triggers and the mechanisms of β-cell death are complex and not fully understood. The Hippo pathway plays a key role in organ size and development through the regulation of proliferation, apoptosis and differentiation. In the present thesis, I investigated the role of Hippo signaling pathway components including NF2, YAP and LATS2 in pancreatic β-cells in normal physiological as well as in diabetic state.

NF2 is an upstream regulator of the Hippo signaling pathway. I showed that NF2 was expressed in both INS-1E cells and primary human islets. Loss of NF2 in pancreatic β-cells could rescue β-cell apoptosis through inhibition of LATS2 activity without compromising β-cell function as well as β-cell functional identity genes.

Transcriptional co-activator YAP is a terminal effector of the Hippo signaling pathway. YAP is not expressed in primary adult β-cells. This could be the reason for the almost non-existing proliferation capacity of human β-cells. Re-expression of the constitutively active form of YAP promoted human β-cell proliferation by regulating transcription factor forkhead box M1 (FOXM1) without altering β-cell function and functional identity genes. Also, YAP re-expression protected β-cells and isolated human islets from apoptosis under diabetogenic conditions. My data showed that YAP overexpression induced small redox proteins thioredoxin-1 and thioredoxin-2 (Trx1/2) at both mRNA and protein levels in both INS-1E cells and human islets and Trx1/2 was required for the anti-apoptotic function of YAP. Together, exogenously introduced YAP functions as pro-proliferative and anti-apoptotic molecule in pancreatic β-cells.

Mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of nutritional status at the cellular and organismic level. While mTORC1 mediates beta cell growth and expansion, its hyper-activation has been observed in pancreatic islets

from animal models of type 2 diabetes and leads to beta cell loss. My data showed that mTORC1 activity was highly increased in type 2 diabetic islets and in human islets exposed to increased glucose concentration, while mTORC2 signaling was diminished. Inhibition of mTORC1 by S6K1 selective inhibitor improved glucose-induced insulin secretion and restored mTORC2 activity in type 2 diabetic islets as well as in isolated diabetic islets from high-fat diet treated mice. This suggests elevated mTORC1 activation as striking pathogenic hallmark of type 2 diabetic islets contributing to impaired β-cell function and survival in the presence of metabolic stress.

Large-tumor suppressor 2 (LATS2) is a core component of the Hippo signaling pathway and an endogenous upstream regulator of YAP. My data showed that overexpression of LATS2 itself was sufficient to induce pancreatic β-cell apoptosis and impair β-cell function. Notably, LATS2 induced β-cell apoptosis through activated mechanistic target of rapamycin complex 1 (mTORC1) by suppression of AMP-activated protein kinase (AMPK) signaling. In addition, while LATS2 overexpression was able to further potentiate chemically-induced defective autophagy and subsequent β-cell apoptosis, its silencing rescued β-cell apoptosis. Loss of LATS2 in isolated human islets and β-cells resulted in resistance to apoptosis induced by diabetogenic conditions *in vitro* and improved glycemia and insulin secretion in the multiple-low dose streptozotocin (MLD-STZ) mouse model *in vivo*. My data suggest that LATS2 acts as a pro-apoptotic molecule in pancreatic β-cells and its inhibition could be an important strategy to improve β-cell survival in diabetes.

Taken together, my data highlight the importance of expression and activation of Hippo signaling elements in proliferation, survival and insulin secretion of pancreatic β-cells. My results suggest that understanding the Hippo signaling pathway in pancreatic β-cell physiology and pathology would offer a new sight to prevent β-cell failure in diabetes.

II Zusammenfassung

Das Versagen der insulinproduzierenden β-Zellen im Pankreas sowohl bei Typ 1 als auch Typ 2 Diabetes ist gekennzeichnet durch den Zelltod der β-Zelle durch Apoptose und geht einher mit einem erheblichen Verlust der Insulinsekretion. Somit stellen Strategien, die an den Regelmechanismen der Apoptose sowie an den Funktionsstörungen der Zelle angreifen, einen attraktiven therapeutischen Ansatz für die Behandlung von T1D und T2D dar. Die tatsächlichen Auslöser und die Mechanismen des β-Zelltodes sind komplex und nicht vollständig verstanden. Durch seinen Einfluss auf Proliferation, Apoptose und Zell-Differenzierung, spielt der Hippo-Signalweg eine Schlüsselrolle in der Regulation von Organgröße und –entwicklung. In der vorliegenden Arbeit untersuchte ich die Haupt-Hippo-Signalweg-Komponenten einschließlich NF2, YAP und LATS2 in pankreatischen β-Zellen im normalen physiologischen und diabetischen Zustand.

NF2 ist ein vorgeschalteter Regler des Hippo-Signalweges. Ich zeigte, dass NF2 sowohl in INS-1E-Zellen als auch in primären humanen Inseln exprimiert wurde. Der Verlust von NF2 in pankreatischen β-Zellen könnte die β-Zell-Apoptose durch Hemmung der LATS2-Aktivität retten, ohne die Zellfunktion sowie funktionelle Identitätsgene zu beeinträchtigen.

Der transkriptionale Co-Aktivator YAP ist ein terminaler Effektor des Hippo-Signalwegs. YAP ist in primären adulten β-Zellen ausgeschaltet. Dies könnte die Ursache für die extrem geringe, kaum messbare Proliferationsrate humaner β-Zellen sein. Re-Expression der konstitutiv aktiven Form von YAP förderte β-Zellproliferation durch Regulierung des Transkriptionsfaktors FOXM1, ohne die β-Zell funktion und funktionelle Identitäts-Gene zu beeinflussen. Auch YAP-Re-Expression schützte die β-Zellen vor Apoptose unter diabetogenen Bedingungen. Meine Daten zeigen, dass YAP-Überexpression die Expression der beiden Redox-Proteine Thioredoxin-1 als auch Thioredoxin-2 (Trx1/2) erhöht und dass Trx1/ 2 für die anti-apoptotische Funktion in den β-Zellen erforderlich ist. Zusammengefaßt fungiert exogen eingeführtes YAP als

pro-proliferatives und anti-apoptotisches Molekül in pankreatischen β-Zellen.

Mechanistic target of rapamycin complex 1 (mTORC1) ist ein Hauptregler des Ernährungszustands auf zellulärer und organismischer Ebene. Während mTORC1 das Wachstum und die Expansion von β-Zellen vermittelt, wurde dessen Hyperaktivierung in Pankreasinseln in Typ-2-diabetischen Tiermodellen beobachtet und führt selbst zu einem Verlust der β-Zelle. Meine Daten zeigten, dass die mTORC1-Aktivität in Inselzellen von Patienten mit Typ 2 Diabetes, sowie in humanen Inseln unter erhöhter Glukosekonzentration stark erhöht war, während gleichzeitig die mTORC2-Signalkaskade vermindert war. Die Hemmung von mTORC1 durch den selektiven S6K1-Inhibitor verbesserte die Glucose-induzierte Insulinsekretion und die Wiederherstellung der mTORC2-Aktivität in diabetischen Inselzellen. Dies deutet auf erhöhte mTORC1-Aktivierung als markante pathogene Kennzeichen von Typ-2-Diabetes-Inseln hin, welches zu einer beeinträchtigten β-Zellfunktion und Überleben in Gegenwart von metabolischer Stresskomponenten beiträgt.

Large-tumor suppressor 2 (LATS2) ist eine Hauptkomponente des Hippo-Signalweges und ein endogener vorgeschalteter Regler von YAP. Meine Daten zeigen, dass die Überexpression von LATS2 selbst ausreichend war, um Pankreas-β-Zell-Apoptose zu induzieren und die β-Zellfunktion zu beeinträchtigen. Bemerkenswerterweise induzierte LATS2 die β-Zell-Apoptose durch Aktivierung von mTORC1 und durch Suppression der AMP-aktivierten Proteinkinase (AMPK). Darüber hinaus führte LATS2 Überexpression zur weiteren Potenzierung des Zelltodes im Rahmen einer defekten Autophagie, Demgegenüber konnte das Ausschalten von Lats2 die β-Zelle vor dem apoptotischen Zelltod retten. Der Verlust von LATS2 in isolierten menschlichen Inseln und β-Zellen führte zu einer Resistenz gegen Apoptose und verbesserte Glykämie und Insulinsekretion im diabetischen Maus-Modell. Meine Daten deuten darauf hin, dass LATS2 als ein pro-apoptotisches Molekül in Pankreas-β-Zellen wirkt und seine Hemmung eine wichtige Strategie zur Verbesserung des β-Zellüberlebens im Diabetes sein könnte.

Zusammengefasst zeigen meine Daten die Bedeutung der Expression und Aktivierung

von Hippo-Signalelementen bei der Proliferation, dem Überleben und der Insulinsekretion von pankreatischen β-Zellen. Meine Ergebnisse legen eine völlig neue Sichtweise der Pankreas-β-Zellphysiologie und -pathologie dar und sind daher richtungsweisend für eine neue Strategie zur Verhinderung des β-Zellversagens in der schweren Stoffwechselkrankheit Diabetes.

III Abbreviations

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
AICAR	5-Aminoimidazole-4-carboxamide riboside
AMPK	AMP-activated protein kinase
APCs	Antigen presenting cells
ATG	autophagy-related
Bcl-2	B-cell lymphoma
Bcl-xL	B-cell lymphoma-extra large
CaMKKβ	Ca2+/Calmodulin-dependent kinase kinaseβ
CBS	cystathione β-synthase
CMA	Chaperone-mediated autophagy
DCs	Dendritic cells
FFA	Free fatty acids
FKBP12	FK506-binding protein 12
FRB	FKBP12-rapamycin binding
FOXM1	Forkhead box M1
GCK	Glucokinase
GLUT2	Glucose transporter 2
GSIS	Glucose-stimulated insulin secretion
hIAPP	Human islet amyloid polypeptide
IFN-γ	Interferon gamma
IL-1β	Interleukin 1 beta

JNK	c-Jun NH2-terminal kinase
LATS	Large tumor suppressor
LKB1	Liver kinase B1
MCP1	Monocyte chemoattract protein 1
MST	Mammalian sterile-20-like kinases
mTOR	mammalian/mechanistic target of rapamycin
mSin1	Mitogen-activated-protein-kinase-associated protein 1
NDRG1	N-Myc downstream regulated 1
NF2	Neurofibromatosis 2
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOD	Non-obese diabetic
PKA	Protein kinase A
PKC-α	Protein kinase C-α
ROS	Reactive oxygen species
S6K1	S6 kinase 1
SGK1	serum- and glucocorticoid-induced protein kinase1
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
TAK1	Transforming growth factor β-activated kinase-1
TCA	Tricarboxylic acid
TNFα	Tumor necrosis factor alpha

TNFR	TNF receptor
Trx	Thioredoxin
TSC	Tuberous sclerosis complex
ULK1	Unc-51 like kinase 1
UPS	ubiquitin-proteasome system
WHO	World Health Organization
YAP	Yes-associated protein

1. Introduction

1.1 Pancreas: structure and function

Diabetes is a complex disease of the insulin producing β-cells, which are located in the islets of Langerhans within the pancreas. The pancreas is a large glandular organ that locates in the upper left part of the abdomen behind the stomach and is surrounded by other organs including the small intestine, liver and spleen. Macroscopically, it can be divided into 3 major parts: head, body and tail [1] (Fig.1). The head of the pancreas is located within the C-shaped concavity of the duodenum. The body of the pancreas is located underneath the stomach extending almost horizontally in the medial plane. The tail of the pancreas extends to the hilum of the spleen. The adult human pancreas is about 14-18 cm long, 2-9 cm wide and 2–3 cm thick, weighing 50–100 g [2].

The pancreas plays an essential role in converting the food into fuel for the body's cells. The pancreas itself consists of two structurally and functionally distinct parts. The exocrine compartment is part of the digestive system and responsible for production and secretion of digestive enzymes, whereas the endocrine compartment is responsible for production and secretion of metabolic hormones. The exocrine gland consists of acinar cells and ductal cells. The main role of the acinar cells is to make and secrete digestive enzymes including proteases (such as trypsin and chymotrypsin), amylase, lipase, and nucleases into the duodenum, which break down proteins, fat and carbohydrates, so they can be absorbed into the circulation. The duct cells secrete a bicarbonate rich fluid, which is responsible for maintaining the duodenal pH. The endocrine compartment consists of islets of Langerhans that include endocrine cells, which produce and release important hormones for glucose homeostasis directly into the bloodstream. The islets of Langerhans are 50-200 μm in diameter [3], and constitute around 1-2% of total pancreas mass [4]. Islets consist of at least 5 different types of hormone-producing cells: the α-cells producing glucagon, the β-cells producing insulin, the δ-cells producing somatostatin, the ε-cells producing hunger stimulating hormone ghrelin and the PP-cells producing pancreatic polypeptide [5]. β-cells mainly in the core of the islet constitute around 60-70% of the

islet cells, while α-cells arranged more in a mantel-core pattern (especially in mice) constitute around 10-20% of the islet cells [6]. In humans, β-cells distribution show a ribbon-like pattern or dispersed throughout the islet in a rather unorganized manner [7]. More recently, it was proposed that human islets form trilaminar plates composed of 2 layers of α-cells surrounding a single layer of β-cells [7].

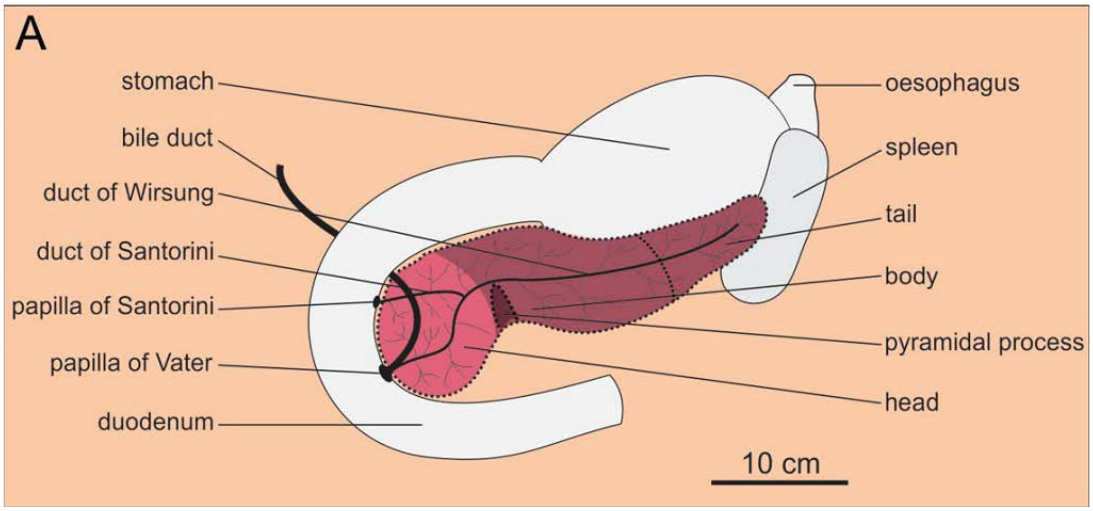

Figure 1. Macroscopic anatomy of the human pancreas (adapted from [7]). Human pancreas consists of the head, the body and the tail.

1.2 Pancreatic β-cells: insulin structure, secretion and function

The main function of β-cells is to secrete insulin in response to increased blood glucose concentration in order to regulate metabolic homeostasis within the body. The mature insulin consists of 21 amino acids of A-chain and 30 amino acids of B-chain bound by disulfide linkage [8]. The secondary structure of the A chain contains two antiparallel α-helices and B chain contains both α-helices and β-sheets. Insulin is an important hormone required for normal metabolism. The secretion of insulin from β-cells is primarily regulated by the glucose concentration in the blood, keeping the blood glucose levels within tight limits [9]. In β-cells, circulating glucose enters into β-cells primarily via glucose transporter 2 (GLUT2) in rodents and GLUT1 in humans on β-cell membrane [10] and then is phosphorylated by rate-limiting glycolytic enzyme glucokinase (GCK). Pyruvate is the metabolic substrate of the glycolysis and oxidized through the tricarboxylic acid (TCA) cycle in the mitochondrial to produce ATP. The increased intracellular ATP/ADP ratio leads to closure of ATP-sensitive SUR1/Kir6.2 potassium (K_{ATP}) channels, depolarizing of the plasma membrane and opening of

L-type voltage-dependent Ca^{2+} channels, resulting in Ca^{2+} influx and insulin-containing granules exocytosis [11] (Fig.2). In addition, many other nutrients as well as metabolic intermediates can also modulate insulin secretion such as amino acids [12], fatty acids [13, 14] and various hormones [11].

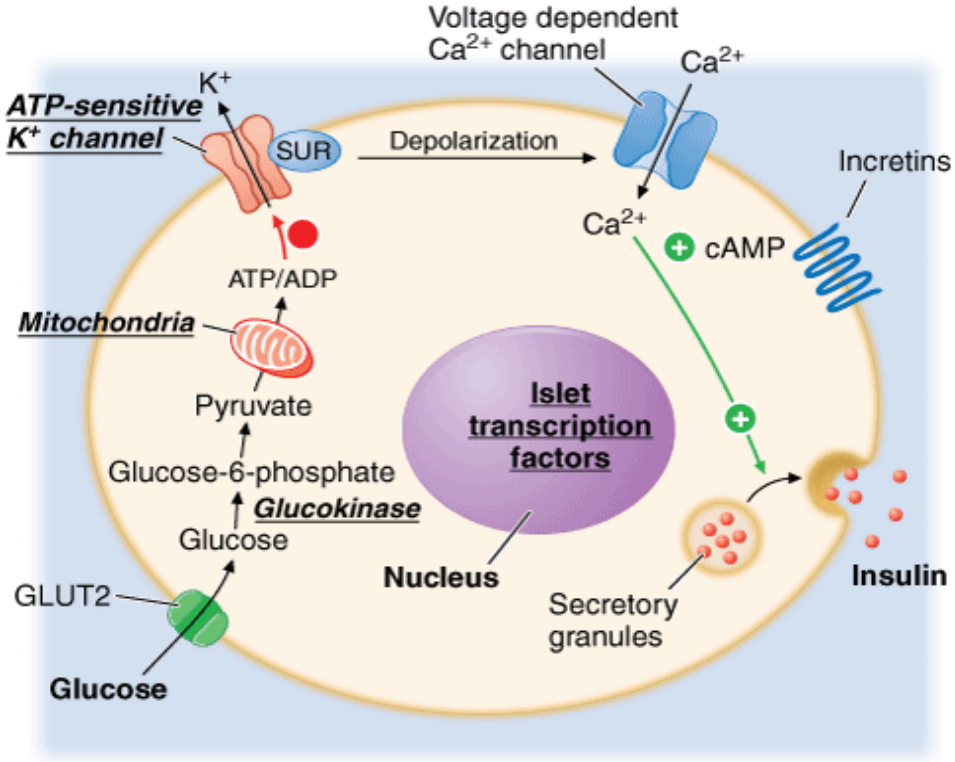

Figure 2. Glucose stimulated insulin secretion in pancreatic β-cells (Adapted from [15]). Glucose is taken up into β-cells through glucose transporter 2 (GLUT2), then phosphorylated to glucose-6-phosphate by glucokinase; glucose-6-phosphate subjected to glycolysis to generate pyruvate in the cytoplasm. Pyruvate is then metabolized by private dehydrogenase and pyruvate carboxylase and enters into the Krebs cycle in the mitochondrial, leading to a rise in the ATP/ADP ratio. Increased ATP/ADP ratio closes the ATP-sensitive SUR1/Kir6.2 potassium channel, results in depolarization of the cell membrane; then voltage-dependent Ca^{2+} channels open, followed by Ca^{2+} influx and elevation of cytosolic free Ca^{2+} concentration; the elevation of Ca^{2+} rapidly increases the rate of insulin exocytosis.

Insulin causes uptake of glucose into liver, muscle and adipocyte, and inhibits glucose production in the liver [16]. Insulin action on adipocytes suppresses lipolysis as well as increases glucose uptake and triglyceride synthesis, regulating glucose homeostasis [17]. Insulin-stimulated glucose uptake in skeletal muscle is used for glycogen synthesis and glycolysis [18]. Insulin action on hepatocytes suppresses gluconeogenesis and glycogenolysisas well as increases glycogen synthesis, leading to suppression of hepatic glucose output [16].

1.3 Diabetes

Diabetes has become a major health problem worldwide. Diabetes is a chronic endocrine disorder and complex metabolic disease characterized by dysregulation of glucose homeostasis and hyperglycemia affecting the body's metabolism, which leads to multiple structural and functional abnormalities in various organs in the body. Serious complications resulting from diabetes include coronary heart disease, stroke, retinopathy, renal failure, peripheral artery disease and neuropathy [19]. Currently, diabetes affects approximately 422 million people worldwide; the number increases rapidly according to the World Health Organization (WHO) [20]. Diabetes is divided into two main types; Type 1 diabetes (T1D) comprising about 5-10% and type 2 diabetes (T2D) comprising about 80-90% of all cases. T1D result from an absolute and T2D from a relative decline in pancreatic β-cell function and/or mass.

1.3.1 Type 1 diabetes

T1D is an autoimmune disease resulting from specific autoimmune destruction of pancreatic islet β-cells [21, 22]. Selective destruction of the β-cells occurs when the islets are abnormally infiltrated with dendritic cells (DCs), macrophages and T lymphocytes, both CD^{4+} and CD^{8+} T lymphocytes are involved [23, 24]. The decreased number of T lymphocytes drives T lymphocyte homeostatic expansion, which results in increased effector/memory T lymphocytes instead of naive T lymphocytes, subsequently kill the islet β-cells [25, 26]. Activated T-cells and macrophages release several mediators such as oxygen free radicals, nitric oxide (NO) and pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (IFN-γ), leading to pancreatic β-cell destruction [11]. The destruction of β-cells diminishes insulin secretion and leads to clinical overt diabetes when the remaining β-cells cannot produce enough insulin to regulate blood glucose levels. *In vitro* cell culture showed that IL-1β or IL-1β plus IFN-γ can activate transcription factor nuclear factor (NF-κB) and c-Jun NH2-terminal kinase (JNK), resulting in β-cell apoptosis [11].

1.3.2 Type 2 diabetes

T2D is defined as a systemic disorder of glucose homeostasis and characterized by peripheral insulin resistance as well as progressive decrease in insulin secretory function and reduced β-cell mass, resulting in the development of chronic β-cell dysfunction and relative insulin deficiency [27, 28]. In people with type 2 diabetes, blood sugar must be controlled either through diet or with oral hypoglycemic drugs or finally with exogenous insulin [29]. There are two steps in developing T2D; Insulin resistance is in general the first step and the second step is β-cell dysfunction. Insulin resistance and other metabolic drivers such as free fatty acids (FFAs) lead to increase in insulin demand from islet β-cells, resulting in increased insulin production by different mechanism such as increased β-cell proliferation and β-cell mass or β-cell hypertrophy. Over time, when islet β-cell compensation for the insulin resistance fails; this leads to a progressive decline in β-cell function, reduced β-cell mass and impaired insulin secretion, T2D manifests [30]. Late stages of T2D are usually accompanied by a decrease up to 60% in pancreatic β-cell mass in T2D patients [27]. Increased pancreatic β-cell apoptosis is the main pathogenic hallmark that contributes to loss of functional β-cell mass [27, 31]. Other proposed mechanisms including β-cell dedifferentiation [32, 33] and failure of adaptive expansion due to impaired proliferation [34] have also emerged as the possible causes of this reduced β-cell mass in T2D.

1.4 Apoptosis in diabetes

Programmed cell death, called apoptosis, is a highly regulated form of cell death, which controls cell homeostasis. Apoptosis is disturbed in many diseases; e.g. it is severely reduced in cancer. Increased apoptosis is the major cause of β-cell loss in the pathogenesis of both T1D and T2D [35-39]. There are two main apoptotic pathways in the mammalian cells: the “extrinsic” (Death receptor-induced) pathway is mediated by cell death receptors such as Fas or TNF receptor (TNFR); the intrinsic pathway (Mitochondrial pathway) is mediated by interactions between pro- and anti-apoptotic mitochondrial proteins of Bcl-2 family members [40]. However, the mechanisms of β-cell apoptosis in diabetes are not well defined.

In T1D, β-cell apoptosis is the result of autoimmune destruction mediated by macrophages and T cells recognizing β-cell antigens [41]. β-cell destruction occurs through activation of immune mediated processes; mononuclear cell and CD^{4+}/CD^{8+} T lymphocytes infiltration in the pancreatic islets and interaction between antigen presenting cells (APCs) and T-cells leads to high local concentrations of pro-inflammatory cytokines, chemokines, ROS and other apoptotic triggers like perforin/granzyme B and Fas/FasL system [35]. The locally produced chemokines and cytokines orchestrate the recruitment and activation of multiple immune cells, and then $CD8^{+}$ T cells directly recognize β-cell fragments on the surface of β-cells, leading to selective β-cell destruction [42]. CD^{4+} and/or CD^{8+} T cells kill β-cells through direct cell contact by perforin/granzyme B system or Fas/FasL pathway, resulting in activation of pro-apoptotic molecule Bid and the mitochondrial pathways of apoptosis [35]. Perforin, Fas or FasL-deficient non-obese diabetic (NOD) mice, animal model of T1D, do not develop insulitis or diabetes [35, 43].

β-cell apoptosis is evident in islets from both lean and obese T2D patients as determined by multiple complementary approaches like *In situ* TUNEL & Capase-3 staining as well as EM-based determination of apoptotic cells [27, 44, 45]. Islet β-cell failure is progressive in T2D and occurs when hyperglycemia established and islets are unable to sustain β-cell compensation for increased insulin demand. There are multiple stimuli including glucotoxicity and lipotoxicity [46, 47], islet amyloid polypeptide (IAPP) [48] and inflammation [49], which can trigger endoplasmic reticulum stress and/or oxidative stress leading to βcell apoptosis [50, 51]. Chronically elevated blood glucose can cause β-cell apoptosis and this may be an important mechanism leading to β-cell deterioration in T2D [30]. High glucose-induced β-cell apoptosis has been reported in several animal models of T2D including the desert gerbil Psammomys obesus [52], Zucker diabetic fatty (ZDF) rat [53], and the domestic cat [54]. Lipotoxicity also induces β-cell apoptosis in T2D. Saturated fatty acids such as palmitate induce β-cell apoptosis through induction of the reactive oxygen species (ROS) and ER stress pathway [51, 55]. Free fatty acids amplify β-cell apoptosis in the presence of high concentrations of glucose, termed as glucolipotoxicity [56]. Islet amyloid is composed of islet amyloid polypeptide (IAPP), which is co-expressed and co-secreted with insulin by β-cells and toxic IAPP oligomers are increased in T2D [27,

57]. Human IAPP is able to induce β-cell apoptosis in vitro [58]. In addition, overexpression of human IAPP in mice also can induce β cell apoptosis via increased ROS production and oxidative stress [59]. Moreover, islet inflammation also contributes to pancreatic β-cell apoptosis in T2D. Macrophage infiltration is elevated in conjunction with increased cytokines and chemokines in the pancreatic islets of T2D patients [60-62] as well as in rodent model of T2D [61, 63]. Studies showed that over-nutrition [64], saturated fatty acids [65], ATP derived from dying cells and endocannabinoids [66] participate in the inflammatory process within pancreatic islets. IL-1β, is a master regulator of inflammation, it promotes β-cell apoptosis and decreases insulin secretion [67]. Increased glucose concentration leads to increased IL-1β production in human islets [68]. High glucose leads to generation of reactive oxygen species (ROS), which indirectly increases IL-1β production in mouse islets [69]. Saturated fatty acids induce IL-1β production leading to downstream to the production of other cytokines and chemokines via the IL-1 receptor 1 (IL-1R1) in both mouse and human islets [70]. Angiotensin II has also been implicated in islet inflammation through increased expression of Monocyte chemo-attractant protein-1 (MCP1) and IL-1β-dependent expression of IL-6 [71, 72].

β-cells are highly sensitive to apoptotic damages induced by multiple stressors such as inflammatory and oxidative assault during the development of diabetes [73]. However, the initial triggers and the mechanisms of β-cell death still remain unclear. Thus, blocking β-cell apoptosis is complicated and difficult to successfully achieve *in vivo*. Identification of intracellular signaling pathways regulating β-cell apoptosis is crucial for better understanding of the molecular changes underlying reduced β-cell mass and would offer a novel targets for therapeutic intervention in diabetes.

The aim of my thesis to identify such novel signaling pathways, which regulate survival/death signals in the β-cells. The important pathways are described in the following chapters.

1.5 Signal transduction pathways in diabetes

1.5.1 AMPK pathway

AMP-activated protein kinase (AMPK) is a highly conserved serine/threonine kinase,

which regulates metabolism and energy balance at both cellular and whole-body levels in obesity, diabetes and the metabolic syndrome [74]. In mammals, AMPK is a heterotrimeric protein complexes consisting of a catalytic α-，regulatory β- and γ-subunits. Each subunit is encoded by a different gene and is expressed as at least two different isoforms. The α subunits (α1 and α2) contain a conserved threonine residue (T172) in the N-terminus which has been shown to essential for AMPK kinase activity [74, 75]. The AMPK β-subunits (β1 and β2) contain a central glycogen-binding domain and a C-terminal domain, which are required for forming a complex with the α- and γ-subunits [76]. The γ-subunits (γ1, γ2 and γ3) contain 4 cystathione β-synthase (CBS) domains, which bind adenine nucleotides (AMP, ATP and ADP) and enhance AMPK kinase activity [77].

In general, AMPK is activated by environmental stress, which causes depletion of cellular ATP, such as hypoxia, heat shock or starvation [78, 79]. It is directly phosphorylated and activated by human tumor suppressor liver kinase B1 (LKB1) at the Thr172 [80]. Ca^{2+}/Calmodulin-dependent kinase kinase β (CaMKKβ) [81-83] and transforming growth factor β-activated kinase-1 (TAK1) [84] are also able to phosphorylate and activate AMPK at the Thr172. An inhibitory site at the Ser485 of α1 subunit has been shown to be phosphorylated by Akt [85, 86], protein kinase A (PKA) [87] or autophosphorylation [88] in various cell types, resulting in reduced AMPK activity. Active AMPK switches on multiple ATP-producing cellular catabolic processes including glucose transport, glycolysis, mitochondria biogenesis and fatty acid oxidation to restore the depleted energy levels [89, 90]. AMPK also can switch off ATP-consuming anabolic processes including protein translation, fatty acid and cholesterol synthesis as well as gluconeogenesis in the liver [91, 92].

Many studies showed that AMPK is dysregulated in the development of insulin resistance and T2D. AMPK activation is able to improve insulin sensitivity and metabolic health [93, 94]. AMPK activity is highly decreased in the skeletal muscle [95] and adipose tissue [96] in obese individuals and inpatients with T2D. Activation of AMPK is able to decrease plasma glucose both by repressing expression of enzymes of gluconeogenesis in the liver and increasing glucose uptake in skeletal muscle and other tissues [74]. 5-Aminoimidazole-4-carboxamide riboside (AICAR), an activator of AMPK, can improve glucose tolerance and reduce hepatic glucose output in ob/ob

mice [97], fa/fa rats [98, 99] and high-fat-fed rats [100]. Currently, Metformin, which can directly activate AMPK, is the drug of first choice for the treatment of T2D [101]. Metformin treatment improves glycemic control and reduces cardiovascular mortality in T2D through activation of AMPK [101]. Another activators of AMPK, thiazolidinediones, are also able to improve glycemic control and insulin sensitivity and used to treat T2D. Taken together, AMPK seems to be an attractive and promising target for the treatment of T2D.

1.5.2 mTOR pathway

The mammalian/mechanistic target of rapamycin (mTOR) is an evolutionary conserved serine/threonine kinase which exists in two biochemically and functionally distinct intracellular complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2) [102] (Fig.3). Diverse mitogens, growth factors, stress, oxygen, nutrients and energy supply stimulate the activation of both mTOR complexes to regulate cell growth, proliferation, development, and viability via controlling multiple down-stream intracellular processes [103, 104]. The mTORC1 and mTORC2 complexes have different sensitivities to rapamycin as well as upstream regulators and downstream regulators. Rapamycin is an allosteric inhibitor of mTOR, it does not directly inhibit mTOR but instead associates with FK506-binding protein 12 (FKBP12), which binds to FKBP12-rapamycin binding (FRB) domain of mTOR and inhibits mTOR activity [105]. mTOCR1 is rapidly dissociated and inhibited by rapamycin [106, 107], while mTORC2 does not interact with FKBP12-rapamycin and is rapamycin-insensitive at short treatment of rapamycin [108, 109]. It has now been shown that long-term treatment of rapamycin can also inhibit mTORC2 signaling in some cell types [108, 110]. mTORC1 is composed of the regulatory-associated protein of mTOR (Raptor), mTOR catalytic subunit, PRAS40 and the adaptor protein mLST8 [111]. mTORC2 is composed of the rapamycin-insensitive protein of mTOR (Rictor), mTOR catalytic subunit, mLST8 and mitogen-activated map kinase-interacting protein 1 (mSin1) [109].

mTORC1 controls protein synthesis through the direct phosphorylation and activation of S6 kinase 1 (S6K1), which in turn phosphorylates the ribosomal protein S6, and through phosphorylation and inactivation of mRNA translation regulator eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) [112]. These two downstream effectors

of mTORC1 whose phosphorylation is inhibited by rapamycin *in vivo*, can be phosphorylated by recombinant mTORC1 *in vitro* [113, 114]. mTORC2 is insensitive to nutrients but activated by growth factors such as insulin through the PI3K/AKT signaling pathway [103]. Activation of mTORC2 phosphorylates the downstream targets N-Myc downstream regulated 1 (NDRG1) and several members of AGC subfamily of kinases including Akt, serum- and glucocorticoid-induced protein kinase1 (SGK1), and protein kinase C-α (PKC-α) to enhance cell proliferation and survival [103, 115]. mTORC2 directly activates and phosphorylates AKT at serine 473 to regulate cellular processes, such as survival, apoptosis and metabolism [103, 108].

mTORC1 is highly activated under energy, nutrients, growth factors and hormones. In response to hypoxia or a low energy state, activated AMPK inhibits mTORC1 through phosphorylation of tuberous sclerosis complex 2 (TSC2), a negative regulator of mTORC1 [116]. Moreover, AMPK also can phosphorylate raptor at serine 792 to induce 14-3-3 binding to raptor, leading to mTORC1 inhibition [117]. Together, AMPK can inhibit mTORC1 activity through either direct or indirect ways.

mTORC1 has been the subject of extensive research in the diabetes field in recent years. Increased glucose, amino acids, pro-inflammatory cytokines and insulin levels in response to nutrient excess promote mTORC1 activity in obese animals [115, 118]. mTORC1 is highly activated in the hypothalamus [119, 120], liver and skeletal muscles [121, 122] of obese and/or high-fat-fed rodents. Of note, mTORC1 promotes insulin resistance in adipose tissue and muscle through mTORC1/S6K1-mediated inhibition of insulin signaling, which reduces glucose uptake and leads to insulin resistance [121, 123]. TSC1，as a tumor suppressor interacts with TSC2 to inactivate Rheb, leading to mTORC1 inhibition [124]. Hypothalamic TSC1 knockout animals improved glycemic control and β-cell function due to mTOR-mediated enhancement of β-cell size and insulin production [125]. Besides several metabolic tissues, mTORC1 is also elevated under conditions of nutrient satiation in the rodent beta cell lines *in vitro* [126] and the pancreatic islets of high fat diet and leptin receptor-deficient *db/db* mice [127].

The mTOR signaling is a key positive regulator of pancreatic β-cell growth and mass. Activation of mTORC1 in β-cells causes a decrease in blood glucose, hyperinsulinemia and improves glucose tolerance through increased β-cell

proliferation and mass in mice [127, 128]. The increased β-cell size and number, which induced by mTORC1 activation in mice, can be reverted by rapamycin [103]. In mice, S6K1 knockout impairs β-cell growth and function through depletion of pancreatic insulin content, hypo-insulinemia, reduced β-cell mass and impaired insulin secretion [129, 130]. Loss of mTORC2 in β-cells causes mild hyperglycemia and glucose intolerance through reduced β-cell mass and impaired insulin production and secretion [131]. But mTORC1 acts as a double edge sword in the regulation of β-cell mass and function in response to nutrient overload and insulin resistance [103]. β-cell specific TSC2-knockout mice with constitutive activation of mTORC1 show increased β-cell mass in the first phase of their life but withaging become hyperglycemic and show severe hypo-insulinemia due to loss of β-cells [127]. Together, these findings suggest that mTORC1 plays important roles in the development of β-cell failure during development and progression of type 2 diabetes.

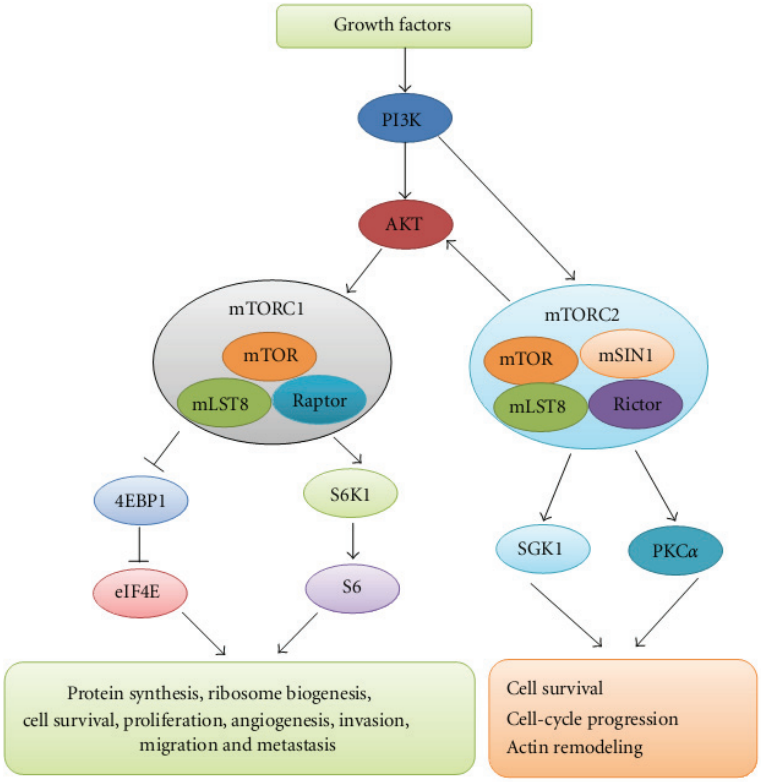

Figure 3. mTOR signaling pathway (adapted from [132]). mTOR complexes includes mTOR complex 1 (mTORC1) and mTOR complex2 (mTORC2). mTORC1 is composed of mTOR, mLST8 and raptor, while mTORC2 is composed of mTOR, mSIN1, mLST8 and rictor. mTORC1 promotes protein synthesis, cell survival and proliferation through phosphorylation of eukaryotic initiation factor 4E-binding protein1 (4EBP1) and ribosomal protein kinase 1 (S6K1), which phosphorylates and activates ribosomal protein S6. mTORC2 functions in cell survival, cell-cycle progression through the regulation of glucocorticoid-induced protein kinase 1 (SGK1)

and protein kinase C α (PKCα).

1.5.3 Autophagy

Autophagy, another form of cell death, is an evolutionary conserved cellular process in eukaryotes that delivers cytoplasmic materials including proteins and organelles to the lysosomes for degradation. It plays important roles in the maintenance of intracellular homeostasis and physiological function of normal tissues [133]. This year (2016), Yoshinori Ohsumi received the Nobel Prize for the discovery of mechanisms for autophagy. There are three major types of autophagy in mammals (Fig.4): macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy is a process, which delivers cargo to the lysosomes through formation and transport of double-membrane vesicles termed autophagosomes. Microautophagy is a process that the small pieces of lysosome are directly engulfed by inward invagination of the lysosomal membrane or late endosomal membrane. CMA involves chaperone-dependent selection of cytoplasmic proteins that are recruited to lysosomes and directly translocated across the lysosome membrane for degradation [134].

As a crucial homoeostatic mechanism, autophagy is activated by cell stress, such as endoplasmic reticulum stress, hypoxia and nutrient starvation [135]. At a basal level, autophagy is important for the clearance of normally occurring misfolded and ubiquitinated proteins. Moreover, autophagy is involved in multiple physiological processes, and its deregulation has been implicated in many human diseases, including cancer, diabetes, neurodegenerative disorders and infectious diseases [134-137]. Among the components of autophagy machinery, the Unc-51 like kinase 1 (ULK1) kinase in mammals, which forms a complex with autophagy-related 13 (ATG13) protein, FIP200 and ATG101, is a key regulator in autophagy initiation [138]. The activation and phosphorylation of ULK1 is essential for autophagy induced by starvation [138]. There are two mechanisms of ULK1-initiated autophagy have been proposed. One mechanism suggests that upon starvation, activated ULK1 directly phosphorylates AMBRA1, a beclin-interacting protein and regulatory component of the PI3K class III complex. The complex then translocates into the endoplasmic reticulum to initiate autophagy [139]. Another mechanism suggests that ULK1 and dAtg1 are able to regulate the actin motor protein myocin II to initiate autophagy [140].

Together, ULK1 may initiate autophagosome generation through both of these two mechanisms.

Recent investigations showed that dysregulated autophagy is also associated with diabetes [141-146]; with altered insulin sensitivity and lipid metabolism [143-145]. Autophagy plays an important role in maintaining pancreatic β-cell mass and function. Autophagy is activated by free fatty acids in pancreatic β-cells and-on the other hand-loss of autophagy in pancreatic β-cells causes reduction in β-cell mass and hypo-insulinemia [141, 142]. Autophagy is highly upregulated in β-cells of high-fat diet fed and *db/db* mice [147]. ATG7 is an E1-like enzyme and essential for autophagy; β-cell-specific Atg7 knockout mice exhibited impaired glucose tolerance and reduced insulin secretion [147]. In addition, lack of autophagy in mice with β cell-specific expression of human islet amyloid polypeptide (hIAPP) results in increased β-cell death and decreased β-cell mass leading to β-cell destruction and development of diabetes [146].

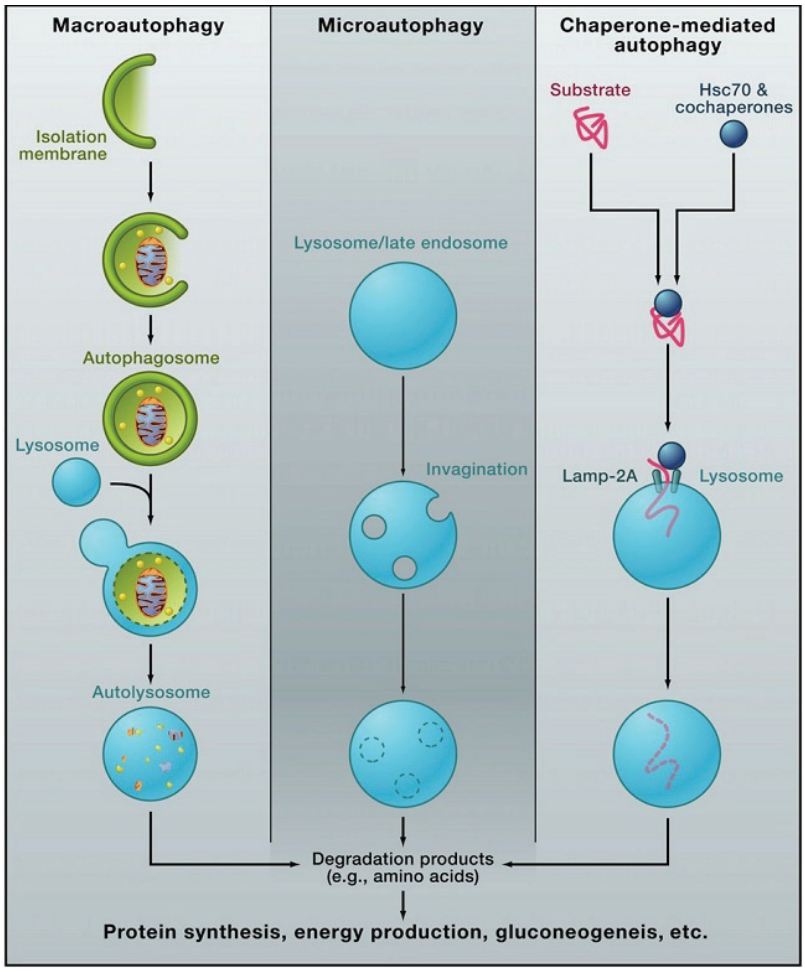

Figure 4. Different types of autophagy (adapted from [134])

Macroautophagy: A portion of cytoplasm, including organelles, is enclosed by phagophore to form an autophagosome. The outer membrane of the autophagosome fuses with the lysosome, and the internal material is degraded in the autolysosome.
Microautophagy: Lysosome itself engulfs small components of the cytoplasm by inward invagination of the lysosomal membrane.
Chaperone-mediated autophagy: Substrate proteins containing a KFERQ-like pentapeptide motif, which is recognized by cytosolic heat shock cognate 70 (Hsc70) and co-chaperones. Then they translocated into the lysosomal lumen and degraded through binding with lysosomal Lamp-2 A.

1.5.4 Crosstalk between AMPK-mTORC1-autophagy

Despite the above described dysregulations of AMPK, mTORC1 and autophagy in diabetes, there is a crosstalk between the 3 pathways (Fig. 5). Both AMPK and mTORC1 can regulate autophagy. Under low-energy conditions, autophagy is induced by activated AMPK or by inactivated mTORC1 signaling. ULK1, as a regulator of autophagy, is directly regulated by energy and nutrient-sensing kinase AMPK and mTORC1 [148, 149]. Under nutrient starvation, the activated AMPK inhibits mTORC1, leading to ULK1-AMPK interaction, then AMPK phosphorylates and activates ULK1 which initiates autophagy [148]. Activated AMPK directly phosphorylates ULK1 at multiple sites including S317, S555 and S777, while mTORC1 phosphorylates ULK1 at the site S757 and disrupts the interaction between AMPK and ULK1 [148, 150,151].

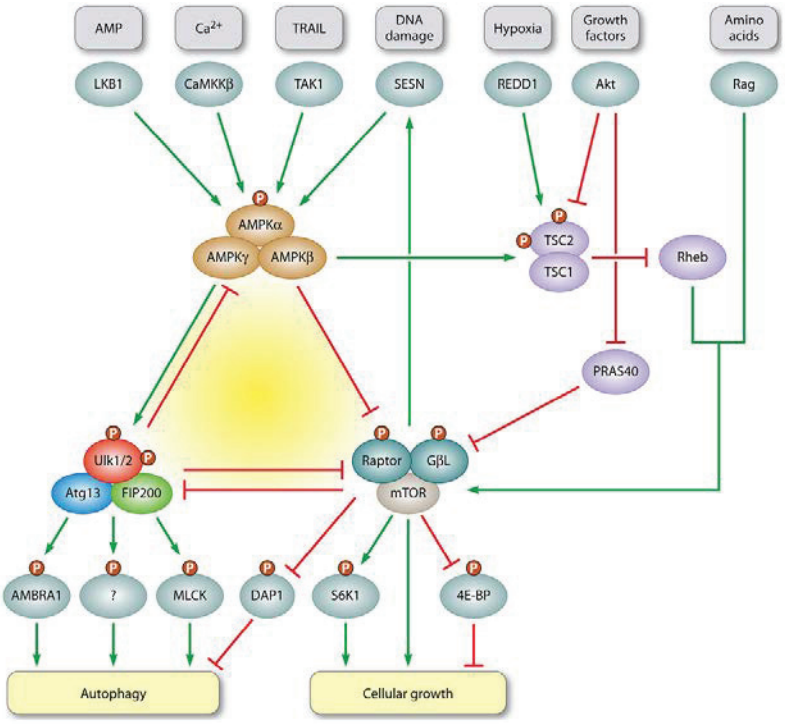

Figure 5. Cross talk between AMPK-mTORC1-autophagy (adapted from [152]). AMPK and mTORC1 oppositely regulate autophagy through phosphorylation of ULK1. Under growth factors and nutrients conditions, mTORC1 activity is positively regulated through the PI3K-Akt pathway, which inhibits TSC1/2 or PRAS40. Activated mTORC1 inhibits autophagy induction by suppressing ULK1 activity or inhibition of DAPI. Under low-energy conditions, AMPK positively regulates autophagy induction through inhibition of MTORC1 or phosphorylation of ULK1.

1.6. Hippo Signaling Pathway

The Hippo pathway plays a key role in organ size control and embryonic development through the regulation of cell proliferation, cell apoptosis and differentiation [153]. The Hippo pathway components include Hippo (Hpo/ MST1/2 in mammals), Salvador (Sav, Sav1/ WW45 in mammals), Warts (Wts/ LATS1/2 in mammals) and Yorkie (Yki/ YAP in mammals) and are highly conserved in both *Drosophila* and mammals [154]. The regulation of the Hippo pathway is complex that may interconnect and cooperate with other signaling pathwaysinvolved in cell survival and proliferation. In mammals, when the Hippo pathway is on, MST1/2 kinase complexes with a scaffold protein, Sav1, phosphorylates and activates the LATS1/2 kinase. Then the adaptor proteins MOB1A and MOB1B bind and activate LATS1/2 kinase [155]. Activated LAST1/2 together with MOB1A/B subsequently phosphorylate and inactivate transcriptional co-activators YAP and TAZ, which leads to YAP and TAZ cytoplasmic retention and inactivation by their interaction with 14-3-3 proteins. Upon multiple additional phosphorylations, YAP and TAZ is marked for degradation by ubiquitin-proteasome system (UPS) [156-158] (Fig.6). When the Hippo pathway is off, the kinases MST1/2 and LATS1/2 are inactive, the YAP/TAZ will be translocated into the nucleus and interact with TEAD1-4 and other transcription factors leading to gene transcription. Upstream, several components have also been implicated by *drosophila* and mammalian genetic studies, including Neurofibromatosis 2 (NF2, also known as merlin), Rassf, FRMD6 and Fat4 [159, 160].

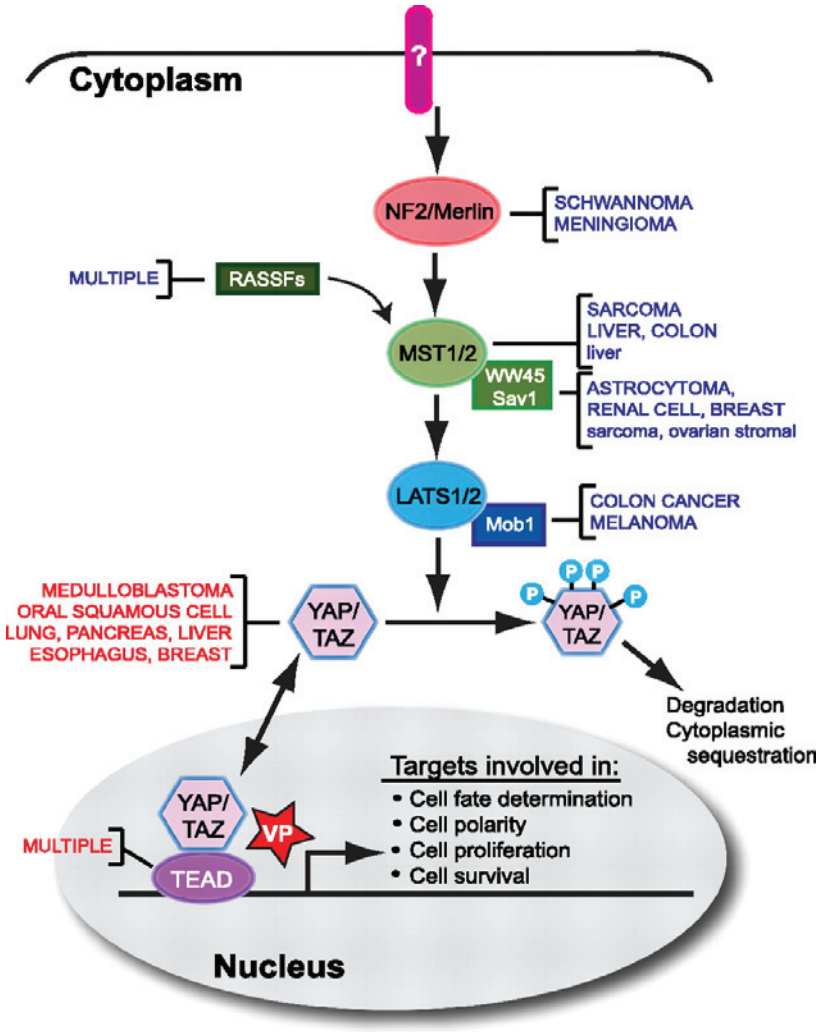

Figure 6. The mammalian Hippo pathway (adapted from [161]). MST1/2, Sav1, LATS1/2, MOB1A/B and Yap/TAZ are the core components of the Hippo pathway. NF2 and Ras association domain family (RASSF) regulate MST1/2 activity. Activated MST1/2 complexes with Sav1 phosphorylates and activates LATS1/2. LATS1/2 interacts with MOB1A/B to phosphorylate and inactivate YAP/TAZ. When the Hippo pathway is on, the phosphorylated YAP and TAZ stay in the cytoplasm and subsequently degraded by the ubiquitin-proteasome system (UPS). When the Hippo pathway is off, the un-phosphorylated YAP/TAZ translocate into the nucleus and bind to TEAD family transcription factors, resulting in the gene transcription of multiple genes.

1.6.1 Neurofibromin 2 (NF2/Merlin)

NF2 is a tumor suppressor protein, which belongs to the ezrin-radixin-moesin (ERM) family of actin-binding proteins and controls cell proliferation and survival though regulation of multiple signaling such as receptor tyrosine kinase (RTK), mTOR, PI3K/AKT and Hippo pathways [162-165]. NF2 is highly conserved in mammals and plays a key role in organ size control and development through the regulation of cell

proliferation and apoptosis [153]. The activity of NF2 is regulated by phosphorylation, which leads to functional inactivation [166, 167] or AKT-dependent degradation [168]. NF2 is directly phosphorylated at Ser10, Thr230 and Ser315 by AKT, resulting in its degradation by ubiquitination [168, 169]. The protein kinase A is also able to phosphorylate NF2 at Ser10 [170] and Ser518 [171], leads to regulation of actin cytoskeleton dynamics or inactivation of NF2 tumor suppressor activity. P-21 activated kinase (PAK) is able to phosphorylate NF2 at Ser518 and inhibit NF2 activity [172]. In contrast, myosin phosphatase (MYPT1-PP1δ) dephosphorylates Ser518 and induces NF2 tumor suppressor activity [166].

Loss of NF2 leads to cell proliferation through activation of PI3K/AKT pathway in human schwannomas and meningiomas [173-175]. Activation of NF2 induced cell apoptosis and blocked tumor initiation through inhibition of mTORC1 signaling [176, 177]. Loss of NF2 activates mTORC1 signaling in malignant mesothelioma: this NF2-deficient phenotype can be reverted by rapamycin [176]. In contrast to mTORC1, NF2 activates mTORC2 in response to growth factor stimulation in Schwann and arachnoidal cells [165].

NF2 is an upstream regulator of the Hippo pathway; it regulates YAP localization and inhibits YAP co-transcriptional activity *in vitro* and *in vivo* [157]. Liver-specific NF2 knockout mice showed hepatocellular carcinoma and bile duct hamartoma. This phenotype is blocked by loss of YAP [178, 179]. NF2 negatively regulates Hippo signaling at two cellular locations, the nucleus and cell cortex. NF2 suppresses tumorigenesis by migrating into the nucleus where it binds and inhibits the E3 ubiquitin ligase CRL4 and through that controls a subset of Hippo pathway target genes [180, 181]. Overexpression of NF2 reduces expression of a group of genes regulated by YAP [180]. Additional studies showed that CRL4 is able to promote LATS1 proteosomal degradation and inhibit LATS2 kinase activity, resulting in decreased YAP phosphorylation [182]. At the cell cortex, NF2 initiates the Hippo signaling pathway through either MST1/2 or LATS1/2 and YAP [163, 178]. NF2 can interact with Kibra and expanded (Ex) to activate the Hippo kinase cascade through direct binding to the Hpo-Sav complex and thus Yki phophorylation [163]. NF2 also functions with Kibra and Sav1 to stimulate Hippo signaling in a Sav1-dependent manner in mammals [178]. NF2 promotes MST1 activation in cardiomyocytes in

mammals [183]. In addition, recent studies show that in both *drosophila* and mammals, NF2 activates Hippo pathway through recruiting the effector kinase Wts/LATS to plasma membranes for phosphorylation by Hpo/MST without altering intrinsic Hpo/MST kinase activities [184]. NF2-deficient mouse Schwann cells have decreased LATS2 phosphorylation and re-expression of NF2 can rescue LATS2's plasma membrane localization [184].

1.6.2 Mammalian Sterile-20-like kinase 1/2 (MST1/2)

The mammalian sterile-20-like (MST) kinase family, which belongs to the class II germinal center kinases (GCK) family of kinases, includes MST1 (also called STK4), MST2 (also called STK3), MST3 (also called STK24), MST4 and YSK1 (also called STK25 or SOK1 in mammals) [185]. The five MST kinases can be divided into two subgroups: MST1/2 and MST3/4/YSK1 [185]. MST1 and MST2 are the core components in the canonical Hippo pathway in mammals and control organ size [185-187]. MST1 and MST2 are highly homologous in mammals. MST1/2 contains a kinase catalytic domain in the N-terminal, an auto-inhibitory domain and a coild-coil dimerization domain known as a SARAH domain in the C-terminal [188] (Fig.7). Two caspase-cleavage sites at D326 and D349 in MST1/2 are located between the kinase and the auto-inhibitory domain [189]. Thus, caspase-mediated cleavage results in removal of the autoinhibitory and SARSH domains and activation of MST1/2 during apoptosis [189, 190]. MST1 and MST2 have been shown to form homodimers and the SARSH domain is required for homodimerization of MST1 and MST2 [188, 191, 192]. Hwang et al show that the SARSH domain of each monomer comprises two helices; a short N-terminal 3_{10} helix (amino acids (aa). 433-437) h1 and a long C-terminal helix (aa.441-480) h2 [193]. In addition, the homodimer interface is mainly stabilized by hydrophobic and hydrogen bonding interactions [193].

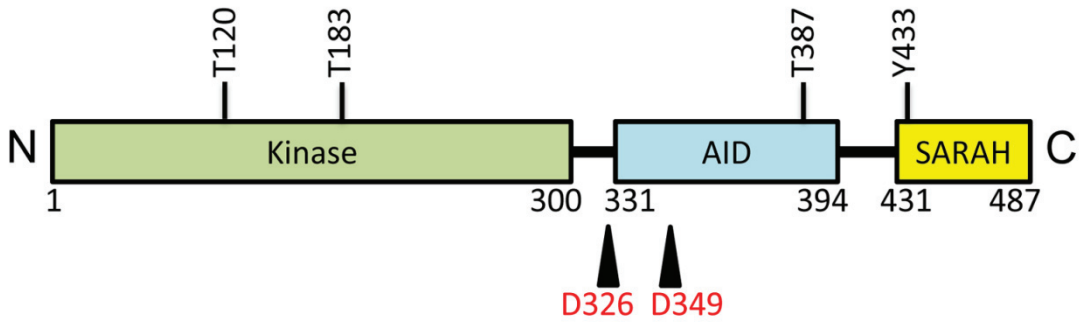

Figure 7. The structure of MST1/2 (adapted from [194]). The MST1/2 is composed of N-terminal kinase domain, a C-terminal SARAH domain and an autoinhibitory domain (AID). MST1 has four established phosphorylation sites (T120, T183, T387 and Y443) and two caspase cleavage sites (D326 and D349).

MST1 and MST2 are activated by diverse apoptotic and stress stimuli, including UV radiation, hydrogen peroxide, staurosporine, retinoic acid, okadaic acid, cytokines, e.g. TNF-α, and DNA-damaging drugs [194]. MST1 and MST2 are auto-phosphorylated at multiple sites which results in their activation [194]. MST1 and MST2 can be phosphorylated by protein kinases such as AKT and c-Abl [194]. AKT binds and phosphorylates MST1 at Thr120 and Thr387, resulting in suppression of MST1 cleavage and activation [195, 196]. Also, mTOR phosphorylates MST1 at Thr120 and leads to prostate cancer cell growth and survival [197]. In addition, pleckstrin homology domain leucine-rich repeat protein phosphatase 1/2 (PHLPP1/2) interact and dephosphorylate MST1 at Thr387, thereby promoting MST1 activation and inducing cell apoptosis [198]. In response to mitogens and oncogenic Ras, AKT is also able to phosphorylate MST2 at Thr117 and Thr387, which leads to MST2 inactivation through several ways: preventing MST2 interaction with RASSF1A, preventing caspase-mediated cleavage of MST2 and promoting MST2 association with Raf1 [199, 200]. In response to oxidative stress, the tyrosine protein kinase c-Abl was also shown to phosphorylate MST1 at Y433 and MST2 at Y81, leading to MST1 and MST2 activation and cell death [201, 202].

In mammalian cells, MST1 and MST2 are the key components in regulating apoptotic signals through multiple phosphorylation-dependent substrates [153, 185, 203,204]. Such MST1/2 pro-apoptotic downstream targets are LATS1/2, histone H2B, FOXO members, c-Jun-N-terminal kinase (JNK) and caspase-3 [204]. In the canonical Hippo pathway, MST1/2 in complex with its adaptor protein Salvador 1 (Sav1) can

phosphorylate and activate LATS1 and LATS2, which in turn phosphorylate and inactivate transcriptional co-activators YAP and TAZ, resulting in regulation of organ size by suppression of cell proliferation and promotion of cell apoptosis [156-158]. Double knockout of MST1 and MST2 in the liver caused YAP aberrant activation, leading to tissue overgrowth and tumor formation [205-207]. MST1 and MST2 double-knockout embryos die early in gestation and show defects in placental development and decreased cell proliferation [208]. Consistently, MST1/2 deletion in the intestine leads to expansion of undifferentiated stem cell compartment and colonic tumorigenesis [209].

MST1/2 in the mammalian pancreas is critical for the regulation of normal metabolism [210-212]. Pancreas-specific MST1/2 knockout mice exhibit decreased pancreas mass and altered pancreas architecture, suggesting that MST1/2 regulate pancreas development [210]. Moreover, the dephosphorylated nuclear form of YAP is increased in the exocrine compartment [210]. In addition, loss of MST1/2 in the pancreas promotes acinar cells de-differentiation or transdifferentiation into duct-like cells [212]. Recently, our lab found that MST1 is a key mediator of pancreatic β-cell apoptotic signaling and β-cell dysfunction in human and rodent β-cells *in vitro* and *in vivo*[211]. MST1 is able to induce β-cell death through a mitochondrial-dependent pathway and impairs insulin secretion through direct phosphorylation of the beta cell transcription factor PDX1 at T11 [211].

1.6.3 Large Tumor Suppressor 1/2 (LATS1/2)

1.6.3.1 Structure of LATS1 and LATS2

Large tumor suppressor (LATS) was originally identified in *drosophila* (called WARTS) as critical regulator of cell apoptosis [213, 214]. There are two homologues of LATS (LATS1 and LATS2) in mammals. LATS1/2 belong to the NDR family of protein kinases and are involved in tumorigenesis by regulating cell proliferation and apoptosis [215]. In mammals, both LATS1 and LATS2 contain a kinase domain in the c-terminal, which has two conserved Ser/Thr phosphorylation sites (LATS1: S909 and T1079; LATS2：S871 and T1041. Serine is the site of auto-phosphorylation and threonine is phosphorylated by both MST1 and MST2), a protein binding domain (PBD), two LATS conserved domains (LCD1 and LCD2), an ubiquitin binding domain (UBA) and at least one PPxY motif (P: Proline, X: any amino acid, Y: Tyrosine) which

binds to WW domains such as YAP and TAZ. As distinct proteins, LATS1 possesses a proline rich region (P-strech) which could be applied for protein-protein interaction [215]. In contrast, LATS2 possesses a seven repeats of alternating proline-alanine residues (PAPA repeat) which may also be involved in distinct protein interactions [215] (Fig.8).

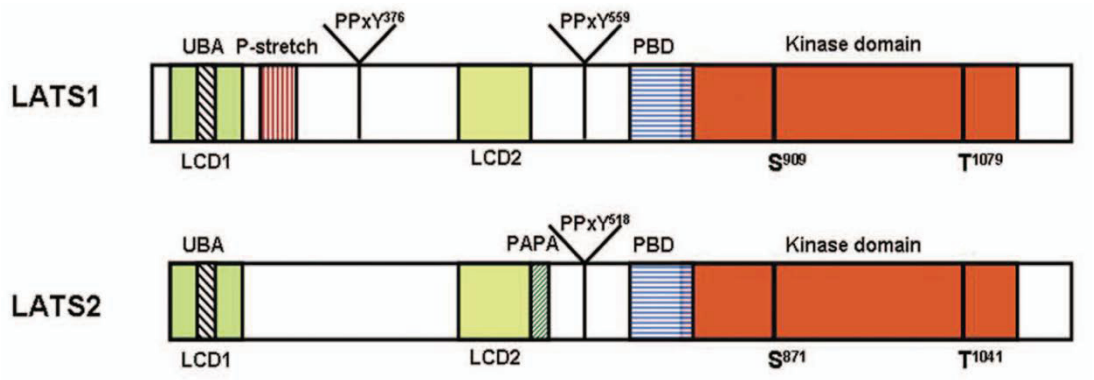

Figure 8. Structure of LATS1/2 in mammals (adapted from [215]). LATS1 and LATS2 share several conserved domains including a C-terminal Ser/Thr kinase domain (LATS1: amino acids (aa.) 708-1130; LATS2: aa.670-1108), a protein binding domain (PBD) (LATS1: aa.656-758; LATS2: aa.618-720), two LATS conserved domains (LCD1 and LCD2) (LATS1: aa.13-167 and aa.458-523; LATS2: aa.1-160 and aa.403-463, respectively) and an ubiquitin binding domain (UBA) (LATS1:aa.101-138; LATS2: aa.99-133). In addition, LATS1 has two PPxY motifs (Y376 and Y559) and LATS2 has one PPxY motif (Y518). As unique features, LATS1 possesses a P-stretch (aa.236-266), while LATS2 possesses a PAPA repeat (aa.467-480).

1.6.3.2 Regulation of LATS activity

There are several established mechanisms by which LATS kinase activity is regulated. These include regulation of expression both at the transcription and protein level, post-translational modifications as well as its sub-cellular localization.

At the transcription level, transcription factor P53 induces LATS2 transcription in response to nocodazole, which specifically recruited to a p53-response element within the promoter region of LATS2 [216]. In addition to nocodazole, oncogenic H-Ras is able to foster LATS2 transcriptional up-regulation through p53-dependent mechanism [217]. For LATS1, the CUX1 transcription factor binds the immediate promoter of LATS1 and induces LATS1 expression [218]. At the post-transcription level, multiple lines of evidence show that miRNAs such as miRNA-31, miRNA-372 and miRNA-373

inhibit LATS2 expression at both the mRNA and protein levels in various cell lines suggesting the existenceof miRNA-regulatory network for regulation of LATS2 at post-transcriptional levels [219-222]. LATS1 is also a downstream target of miRNA-21 [223]. At the protein level, ROS-PKCδ signaling decreases LATS1 in human senescent cells [224]. In addition, the kinase NUAK1 is able to phosphorylate LATS1 at S464 to decrease LATS1 protein stability [225]. For LATS2, the signaling of PKCδ, NUAK1 and H-Ras are shown to modulate LATS2 protein stability [215, 217]. LATS1 and LATS2 protein levels are also regulated by UPS at post-translational level. E3 ubiquitin ligases such as NEDD4 [226] and WWP1 [227] ubiquitinate and down-regulate LATS1. E3 ubiquitin ligase SIAH2 destabilizes and decreases the steady-state of endogenous LATS2 protein through proteosomal or lysosomal pathways [228]. Kibra can stabilize LATS2 by inhibiting its ubiquitination [229]. In the canonical Hippo pathway, LATS1/2 is phosphorylated by both MST1/2 at the site of T1079 and T1041, respectively [187]. LATS1/2 phosphorylation is increased by up-regulation of NF2, which is amplified by co-expression of NF2 and other Hippo regulatory adaptor protein Kibra [163, 230]. Moreover, LATS2 is phosphorylated by mitotic kinase Aurora-A at the site of S83 during mitosis; this phosphorylation is responsible for LATS2 localization to the centrosome during cell cycle [231].

1.6.3.3 Functions of LATS

LATS1 and LATS2, as tumor suppressors, play important roles in cell proliferation, apoptosis and migration. Overexpression of LATS1 or LATS2 dramatically inhibits both cell proliferation and anchorage-independent growth in various cell lines [157, 215, 232-234]. Conversely, knockdown of LATS1 in mammalian cells increases cell proliferation [235], downregulation of LATS2 leads to contact inhibition of growth in mouse embryonic fibroblasts (MEFs) and LATS2 knockout mice are embryonically lethal [236, 237]. In the context of Hippo signaling, YAP/TAZ inhibitory phosphorylation mediated by LATS1 and LATS2 is the principal mechanism initiating pro-apoptotic network which fine-tunes apoptosis during development or as protective tumor-suppressor mechanisms [210]. LATS1 inhibits cell proliferation by blocking the G_2/M transition [238], whereas LATS2 inhibits cell proliferation through blocking both G_1/S and G/M transition [215]. Also, LATS1 induces apoptosis through upregulation of

pro-apoptotic proteins P53 and Bax [215], whereas LATS2 induces apoptosis through downregulation of anti-apoptotic proteins such as Bcl-2 and Bcl-xL [217, 239]. LATS1/2 depletion in the early preimplantation embryo prevents inner cell mass lineage differentiation [240]. Additional deletion of LATS2 in liver-specific Sav1 knockout mouse accelerated tumorigenesis highlighting its critical role as tumor-suppressor player [241]. Consistently, knockdown of LATS2 induces oncogenic H-Ras-v12-dependent cell migration [217].

LATS kinases have been suggested as regulator of cytoskeletal dynamics. LATS1 interacts with various cytoskeletal proteins including Zyxin [242] and LIMK1 [243] to regulate actin dynamics. LATS2 associates with the microtubule-associated Ajuba, recruiting γ-tubulin to the centrosome [244]. LATS2 regulates the transcription of different genes, such as probasin and PSA [245]. In addition to transcriptional regulation, LATS2 directly regulates steady-state of multiple proteins such as p53, Aurora-B and PLK1, as well as mitochondrial proteins Bcl-2, Bcl-xL and Bax [236, 246]. LATS2, but not LATS1 regulates both growth and death in cardiac myocytes, which is dictated by MST1 [247]. This suggests that the function of LATS1 and LATS2 may be cell type-dependent in mammalian cells.

Together, both LATS1 and LATS2 play a critical role in cell proliferation, apoptosis, and migration, by modulating transcriptional regulation and protein stability of different substrates.

1.6.4 Yes-associated protein (YAP)

YAP is a transcriptional co-activator and a major effector of the mammalian Hippo pathway [187]. There are two major isoforms of YAP which are generated by differential splicing, named YAP1 and YAP2 [248]. Both YAP1 and YAP2 contain a proline-rich region at the very amino terminus, TEAD binding domain, an SH3-binding motif, a transcriptional activation domain, a PDZ domain-binding motif at the C-terminal (Fig.9) [249]. The difference between YAP1 and YAP2 is that YAP1 has one WW domain, while YAP2 has two WW domains [249].

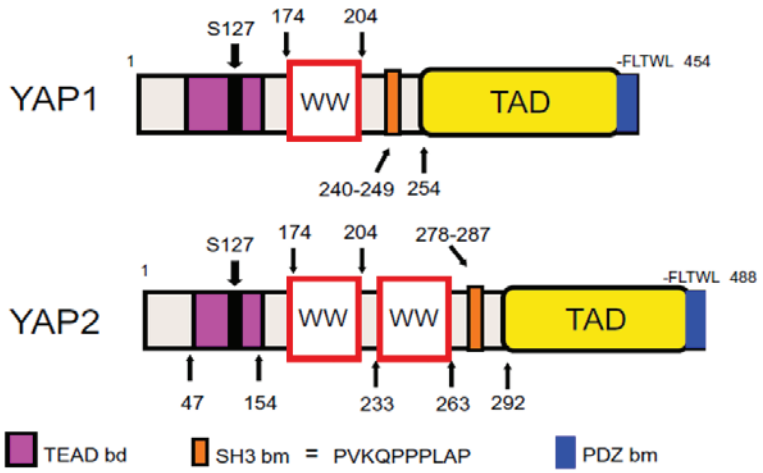

Figure 9. Modular structures of Yap1 and Yap2 (adapted from [249]). The Yap protein contains TEAD binding domain (TEAD bd), a single WW domain in the YAP1 isoform and two WW domains in the YAP2 isoform, SH3 domain-binding motif (SH3 bm), transcriptional activation domain (TAD) and PDZ domain-binding motif (PDZ bm).

In mammals, YAP is directly phosphorylated by LATS1/2 kinase on five HXRXXS consensus motifs at the sites of S61, S109, S127, S164 and S381 [250]. Moreover, S127 and S381 are key phosphorylation sites, which are critical for suppressing YAP nuclear translocation and activity [158, 250]. Phosphorylated YAP and its paralog TAZ by LATS1/2 bind 14-3-3 protein, which sequesters YAP in the cytoplasm and ultimately leads to ubiquitin-dependent degradation [157, 251]. Conversely, un-phosphorylated YAP and TAZ is translocated into the nucleus and interact with several transcription factors including TEAD family, P73 and Runx2, resulting in cell proliferation and survival in many cell types [187]. Moreover, AMPK directly phosphorylates YAP on S94 to disrupt YAP-TEAD complex, leading to inhibition of cell growth [252]. As mentioned before, the Hippo pathway plays a critical role in controlling organ size by regulating cell proliferation and cell apoptosis, and this regulation is mediated by the expression level or localization of YAP. Tissue-specific overexpression of YAP in the mouse liver or heart showed liver or heart overgrowth and eventually leads to liver or heart tumors as a result of increased cell proliferation [154, 253, 254]. Mice with liver-specific ablation of Sav1 show increased liver size and expansion of hepatic progenitor cells through YAP up-regulation and its localization to the nucleus [255]. Heart-specific Sav1 knockout mice have enlarged hearts and cardiomegaly by similar mechanism involving YAP aberrant activity [256]. Consistently, liver-specific MST1 and/or MST2 knockout mouse show loss of S127 YAP inhibitory

phosphorylation, which results in liver size expansion and hepatocellular carcinoma [205, 207].

1.6.5 The Hippo pathway in pancreas development and islet biology

The Hippo pathway has been studied in context of pancreas development [210, 212,257], although much less is known about the role and expression of Hippo signaling components in the adult β-cell at normal and disease states. Our laboratory has recently identified MST1, the key core component of Hippo signaling, as a disease modulator in isolated human and rodent islets, in human pancreatic autopsy tissue and in animal models of diabetes [211]. All applied diabetic stimuli, i.e. free fatty acids, elevated glucose, inflammatory cytokines, oxidative or ER stress induces MST1 activation and MST1 mediates β-cell apoptosis and dysfunction. Mechanistically, MST1 impairs β-cell function and survival at least through two mechanisms: (i) MST1 specifically activates the mitochondrial pathway of cell death through up-regulation of the pro-apoptotic member BIM, which leads to β-cell apoptosis. (ii) MST1 directly phosphorylates the critical β-cell transcription factor PDX1 on Thr11, resulting in ubiquitination and degradation of PDX1, which leads to functional β-cell impairment. Also, Inhibition of MST1 protects from β-cell death *in vitro* in human islets as well as in animal models of diabetes *in vivo*.

At prenatal developmental stage, pancreas proliferation and cell-type specification is regulated by Hippo signaling. As major downstream effectors, TEAD and its co-activator YAP play a crucial role in the expansion of pancreatic progenitors by controlling key pancreatic signaling mediators and transcription factors in the embryonic phase of pancreas development [257]. Consistently, YAP depletion is sufficient to block pancreatic progenitor cell proliferation [258]. YAP expression is decreased as pancreas development proceeds, which subsequently switches off in the mature endocrine but not in the exocrine cells [210, 212]. This correlates with the extremely low rate of β-cell proliferation and β-cell quiescence. Thus, while YAP signals are disconnected from the core Hippo kinases in mature islets, MST1/2 and LATS1/2 are still expressed and are able to activate Hippo signaling in the absence of YAP suggesting the presence of alternative Hippo down-stream effector(s). Notably, lack of MST1 alone [211] or both MST1 and MST2 [210] are not sufficient to drive pancreatic β-cells out of quiescence and induce β-cell proliferation. Absence of YAP

as critical signaling element of the Hippo pathway may explain the lack of endocrine cell proliferation in this context. Conversely, pancreas-specific MST1/2 knockout is sufficient to dramatically increase ductal and acinar proliferation, due to aberrant YAP activity in the exocrine compartments [210]. Overexpression of a constitutively active form of YAP increases proliferation of insulin-producing β-cells without negatively affecting β-cell differentiation and β-cell function in human cadaver islets [259].

1.7 Aim of thesis

Apoptosis and loss of function are hallmarks of β-cell failure and the fundamental cause of diabetes [31, 43, 260]. Targeting β-cell apoptosis and dysfunction therefore represents an attractive therapeutic approach forthe treatment of diabetes. The Hippo pathway plays a key role in organ size and development through the regulation of cell proliferation, apoptosis and differentiation [153]. We have been identified MST1, a core component of the Hippo pathway, as a principal regulator of pancreatic β-cell apoptosis and dysfunction in human and rodent β-cells *in vitro* and *in vivo* [211]. Despite the established function of MST1 in β-cell apoptosis and dysfunction, the role of other Hippo components on islet physiology and patho-physiology is not known so far. The aim of this thesis was to investigate the function of β-cell's Hippo signaling components mainly Merlin/NF2, YAP and LATS1/2 and its underlying mechanisms-inorder to get new insights from Hippo signaling in pancreatic β-cells at normal physiology as well as disease-state. I put my major efforts to identify Hippo signaling mechanisms and molecular events that are responsible for cellular and molecular alterations during β-cell failure in diabetes. This knowledge would be important for better understanding of the molecular changes underlying functional β-cell mass loss and development of therapeutic strategies for the treatment of diabetes.

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2. Results

Manuscript I

2.1 Loss of NF2 protects pancreatic β-cells from apoptosis by inhibiting LATS2

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Loss of Merlin/NF2 protects pancreatic β-cells from apoptosis by inhibiting LATS2

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Dear Editor,

A fundamental challenge in treating diabetes is the identification of molecular states that cause β-cell failure in response to pro-diabetic conditions. Both type 1 and type 2 diabetes mellitus result from an absolute or relative decline in pancreatic β-cell insulin secretion and/or mass. Apoptosis and loss of function are hallmarks of β-cell failure and the fundamental cause of diabetes.[1,2] We have recently identified mammalian sterile-20-like kinase (MST1), the key component of Hippo signaling, as a novel regulator of pancreatic β-cell death and dysfunction in human and rodent β-cells *in vitro* as well as in diabetic animal models *in vivo*. MST1 promotes β-cell apoptosis through regulation of multiple downstream targets, such as c-Jun N-terminale Kinase (JNK), caspase-3, histone H2B and mitochondrial Bcl-2 member family.[3] However, the mechanism of upstream regulation of MST1 in the β-cell and the 'β-cell Hippo signaling' cascade has not yet been investigated. Such critical upstream regulator of the Hippo signaling pathway is neurofibromatosis type 2 (NF2) or Merlin, a tumor suppressor protein, which belongs to the ezrin–radixin–moesin family of actin-binding proteins. It is conserved in both Drosophila and mammals, and plays a key role in organ-size control and development through the regulation of cell proliferation and apoptosis.[4] NF2 initiates the Hippo signaling by directly activating MST1/2 kinases,[5] or by recruiting large-tumor suppressor kinase 1/2 (LATS1/2) to membranes for phosphorylation by MST1/2 without altering intrinsic MST1/2 kinase activities.[6] So far, the physiological role of NF2 in the β-cell, and whether its loss would regulate β-cell death and insulin secretion as well as downstream Hippo kinases are not known.

NF2 is expressed in isolated human islets, in the rat insulinoma β-cell line INS-1E (Figure 1) as well as in mouse islets (data not shown). Inhibition of endogenous NF2 by siRNA knockdown rescued INS-1E cells from high glucose- and high-glucose/palmitate-induced apoptosis, as demonstrated by decreased caspase-3 levels and Poly-(ADP-ribose)-polymerase (PARP) cleavage (Figure 1a). Consistently, NF2 silencing protected β-cells from pro-inflammatory cytokines and high-glucose/palmitate-induced apoptosis in isolated human islets (Figure 1a). As NF2 functions upstream of the core Hippo pathway kinase LATS1/2 and MST1/2, we aimed to identify whether NF2 changes LATS1/2 and MST1/2 activities under diabetic conditions. Although NF2 knockdown did not change MST1 hyperactivation under glucotoxic conditions, it remarkably reduced LATS1/2 phosphorylation demonstrating NF2-dependent LATS1/2, but not MST1/2 regulation in β-cells (Figure 1a). This is in line with the recently suggested alternative model of NF2 function through direct binding to LATS proteins.[6] Intriguingly, LATS2 reconstitution followed by NF2 knockdown in INS-1E cells abrogated β-cell protection by NF2 loss, confirming LATS2-dependent action of NF2 in pancreatic β-cells (Figure 1a).

NF2 controls cell survival by integrating signals initiated through cell–cell interactions or extracellular cues by direct suppression of growth regulatory and antiapoptotic pathways, including the mechanistic target of rapamycin (mTOR) signaling.[7] As mTORC1 is a critical pro-survival signal in β-cells whose transient hyperactivation has pleiotropic functions leading to increased β-cell mass,[8] we checked whether NF2 loss has an impact on mTORC1 activity, as represented by downstream substrates S6K and 4EBP1 phosphorylation. Notably, S6K as well as 4EBP1 phosphorylation was highly upregulated by NF2 depletion in both isolated human islets and INS-1E cells, suggesting direct regulation of β-cell antiapoptotic mTORC1 by NF2 (Figure 1b). Despite its critical role in apoptosis inhibition, NF2 depletion neither affects glucose-stimulated insulin secretion, nor insulin gene expression and critical genes involved in glucose sensing and insulin transcription. Thus, NF2-dependent β-cell protection occurred without affecting β-cell function (Figure 1c).

In conclusion, our data show a direct protective effect of NF2 depletion in pancreatic β-cells by inhibiting LATS2 but not MST1 activity, which could rescue β-cells from apoptosis without compromising β-cell function. Also, mTORC1 hyperactivation might be involved in the pro-survival mechanism of NF2 deficiency. The identification of NF2 as the key upstream regulatory and disease-relevant component of the Hippo signaling provides a novel area for potential therapeutic approaches aiming to block β-cell apoptosis in order to restore a functional pancreatic β-cell mass in diabetes.

Conflict of Interest

The authors declare no conflict of interest.

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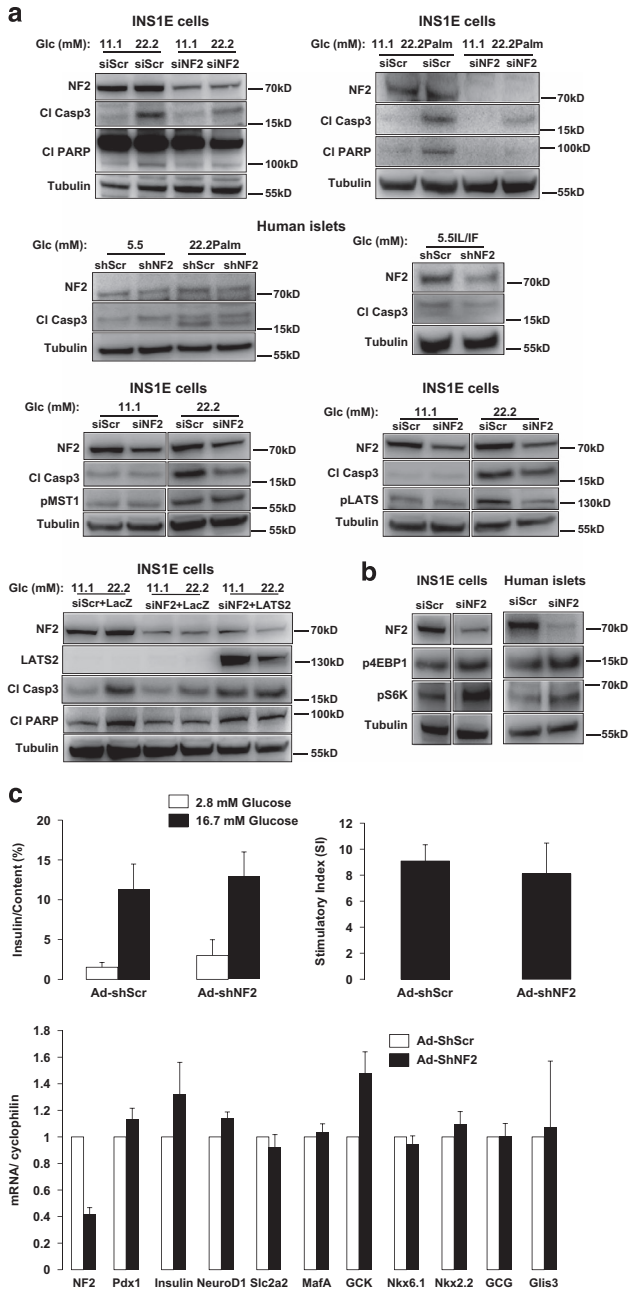

Figure 1 (**a**) INS-1E cells were transfected with either control Scr siRNA (siScr) or NF2 siRNA (siNF2) (both from Dharmacon, Lafayette, CO, USA) and treated with 22.2 mM glucose or the mixture of 22.2 mM glucose and 0.5 mM palmitate (22.2 Palm) for 2 days. Human islets were infected with either control Ad-GFP-Scr or Ad-GFP-hShNF2 adenoviruses (VectorBioLabs, Malvern, PA, USA) for 1 day and then treated with the mixture of 22.2 mM glucose and 0.5 mM palmitate or the pro-inflammatory cytokines 2 ng/ml recombinant human IL-1β and 1000 U/ml IFNγ (IL/IF) for 3 more days. INS-1E cells were transfected with either control siScr or siNF2 and treated with 22.2 mM glucose for 2 days. INS-1E cells were transfected with either control siScr or siNF2 for 1 day and then infected with either Ad-LacZ control or Ad-LATS2 adenoviruses (VectorBiolab) and then treated with 22.2 mM glucose for another day. (**b**) INS-1E cells and isolated human islets were transfected with either control siScr or siNF2. Cleaved capase-3, cleaved PARP, NF2, pMST1, pLATS1/2, LATS2, pS6K and p4EBP1 were analyzed by western blotting. Western blots show representative results from three independent experiments (INS-1E) from three different donors (human islets). Tubulin was used as loading control. (**c**) Human islets were infected with either control Ad-GFP-Scr or Ad-GFP-hshNF2 adenoviruses (VectorBiolab) for 2 days. Insulin secretion during 1 h incubation with 2.8 mM (basal) and 16.7 mM glucose (stimulated), normalized to insulin content. The insulin stimulatory index denotes the ratio of secreted insulin during 1 h incubation with 16.7 and 2.8 mM glucose. Pooled data from three independent experiments from three different donors (human islets). Quantitative RT-PCR for *NF2, Insulin, Pdx1, NeuroD1, Slc2a2, MafA, GCK, Nkx6.1, Nkx2.2, GCG and Glis3*. For analysis, we used the AppliedBiosystems StepOne Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) with TaqMan(R) Fast Universal PCR Master Mix for TaqMan assays (AppliedBiosystems). Pooled data from four independent experiments from four different donors (human islets)

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Manuscript II

2.2 Pro-proliferative and anti-apoptotic action of exogenously introduced YAP in pancreatic β-cells

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Designed and performed experiments (Figures 1-4), analyzed data and wrote the paper.

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RESEARCH ARTICLE

Proproliferative and antiapoptotic action of exogenously introduced YAP in pancreatic β cells

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Loss of functional pancreatic β cells is a hallmark of both type 1 and 2 diabetes. Identifying the pathways that promote β cell proliferation and/or block β cell apoptosis is a potential strategy for diabetes therapy. The transcriptional coactivator Yes-associated protein (YAP), a major downstream effector of the Hippo signaling pathway, is a key regulator of organ size and tissue homeostasis by modulating cell proliferation and apoptosis. YAP is not expressed in mature primary human and mouse β cells. We aimed to identify whether reexpression of a constitutively active form of YAP promotes β cell proliferation/survival. Overexpression of YAP remarkably induced β cell proliferation in isolated human islets, while β cell function and functional identity genes were fully preserved. The transcription factor forkhead box M1 (FOXM1) was upregulated upon YAP overexpression and necessary for YAP-dependent β cell proliferation. YAP overexpression protected β cells from apoptosis triggered by multiple diabetic conditions. The small redox proteins thioredoxin-1 and thioredoxin-2 (Trx1/2) were upregulated by YAP; disruption of the Trx system revealed that Trx1/2 was required for the antiapoptotic action of YAP in insulin-producing β cells. Our data show the robust proproliferative and antiapoptotic function of YAP in pancreatic β cells. YAP reconstitution may represent a disease-modifying approach to restore a functional β cell mass in diabetes.

Introduction

β Cell failure (loss of β cell function and mass) is a hallmark of both type 1 and 2 diabetes (T1D/T2D). Over the past 20 years, little progress has been made in identifying efficient strategies to stop β cell failure. The further decline in β cell function during current therapies highlights the need for improved therapeutic approaches as well as better understanding of the molecular changes underlying functional β cell loss in diabetes. Apoptosis of insulin-producing β cells is the fundamental cause of T1D and a contributing factor to the reduced β cell mass in T2D (1–4). Given the varied and enigmatic nature of the causes of β cell failure, inhibition of apoptosis and/or enhancement of β cell regenerative capacity by augmenting β cell proliferation represent attractive therapeutic approaches to the treatment of diabetes (5).

Identification of signaling molecules that regulate both β cell apoptosis and proliferation, together with an in-depth knowledge of their mechanisms of action is a prerequisite for the discovery of new drugs for β cell–directed therapies in diabetes. Characterization of signaling cascades such as PI3K-AKT, MAPK, and Wnt has particularly contributed to the understanding of β cell pathogenesis in diabetes (6–12). A more recently discovered signal is the Hippo pathway that was proposed to regulate organ size. Initially defined in Drosophila, the conservation of this pathway in mammals has been firmly established, where it consists of the core kinase complexes mammalian sterile 20–like kinases (MST1/2) and large-tumor suppressors (LATS1/2), adaptor proteins (SAV1 for MST1/2 and MOB1 for LATS1/2), and downstream transcriptional coactivators (YAP and TAZ). MST1/2 kinases phosphorylate and activate LATS1/2 kinases. Active LATS1/2 phosphorylates YAP at serine 127 (S127) and provides the docking site for the 14-3-3 protein, which sequesters YAP in the cytoplasm and ultimately leads to ubiquitin-dependent degradation that thereby prevents YAP transcriptional activity (13–16). YAP acts mainly

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through TEA domain (TEAD) family transcription factors to promote the expression of target genes required for cell proliferation and survival. The loss of any component of the kinase core results in a YAP-dependent increase in proliferation and resistance to apoptosis in multiple tissues, suggesting Hippo as a powerful tool to regulate organ size (16–18).

Targeting the Hippo signaling pathway has recently emerged as an attractive therapeutic strategy for treatment of various pathological disorders (19–24). Although previously studied in pancreas development (25–27), little is known about the role of Hippo signaling components in the adult β cell in normal and disease states. We have recently identified MST1, the key core component of Hippo signaling, as a principal regulator of pancreatic β cell apoptosis and dysfunction in human and rodent β cells in vitro as well as in diabetic animal models in vivo (28, 29). At prenatal developmental stages, pancreas proliferation and cell-type specification is regulated by Hippo signaling. As major downstream effectors, TEAD and its coactivator YAP play a crucial role in the expansion of pancreatic progenitors by controlling key pancreatic signaling mediators and transcription factors in the embryonic phase of pancreas development (27). Consistently, YAP depletion is sufficient to block pancreatic progenitor cell proliferation (30). However, convincing studies have revealed that YAP is not expressed in terminally differentiated mature primary human and mouse β cells. YAP expression decreases as pancreas development proceeds and eventually switches off in the mature endocrine but not in the exocrine cells (25, 26); this correlates with the extremely low rate of β cell proliferation and β cell quiescence. Thus, while YAP signals are disconnected from the core Hippo kinases in mature islets, MST1/2 and LATS1/2 are still expressed and are able to activate Hippo signaling in the absence of YAP, suggesting the presence of alternative Hippo downstream effector(s). Notably, lack of MST1 alone (28) or both MST1 and MST2 (26) is not sufficient to drive pancreatic β cells out of quiescence and induce β cell proliferation. The absence of YAP as a critical signaling element of the Hippo pathway may explain the lack of endocrine cell proliferation in this context. Conversely, pancreas-specific MST1/2 knockout is sufficient to increase ductal and acinar proliferation, due to aberrant YAP activity in the exocrine compartments (26).

Since YAP has both antiapoptotic and proproliferative activity in other cell types, the reexpression of YAP may provide a strategy directed towards promoting β cell proliferation and protecting from stress-induced conditions relevant to diabetes. In this study, we aimed to identify whether reexpression of a constitutively active form of YAP promotes β cell proliferation in isolated human islets and whether such overexpression would also protect β cells from apoptosis under diabetic conditions.

Results

Overexpression of constitutively active YAP (YAP^{S127A}) promotes human β cell proliferation. Given the proproliferative function of YAP in other cell types, we asked whether reintroduction of the activated form of YAP is sufficient to drive β cell proliferation. Isolated human islets were infected with adenovirus expressing luciferase (Ad-Luc) as a control or Ad-YAP expressing activated YAP with a serine 127 to alanine substitution (YAP^{S127A}) that abolishes MST/LATS-mediated YAP inactivation and provides a constitutively active form of YAP (31). YAP overexpression remarkably increased β cell proliferation in isolated primary human islets seen in multiple different organ donors by significantly increased Ki67-positive (Figure 1, A and B, and Figure 2, D and E) as well as BrdU-positive β cells (Figure 1, C and D, and Figure 2, F and G). Our triple-staining data show that most of the Ki67- and insulin-positive or BrdU- and insulin-positive cells are YAP positive (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/jci.insight.86326DS1), suggesting a cell-autonomous action of YAP on β cell proliferation.

Gene expression profiling showed that YAP potently and coordinately initiates gene programs that promote cell-cycle activity and proliferation in different cell types (32, 33). As many of these YAP-target genes are able to promote β cell proliferation, e.g., the transcription factor forkhead box M1 (FOXM1) (34), their identification as regulators of YAP might provide a molecular explanation of how YAP induces β cell proliferation. To identify the downstream YAP effector and mediator of YAP-induced proliferation in β cells, we investigated expression levels of FOXM1. YAP overexpression significantly induced a 2-fold elevated FOXM1 mRNA expression in human islets (Figure 2A) together with an increase in FOXM1 protein (Figure 2, B and C) levels. To investigate the potential of FOXM1 as a mediator of YAP-induced β cell proliferation, we used a pharmacologic inhibitor of FOXM1 expression, thiostrepton (35, 36). Thiostrepton fully blocked β cell proliferation in the Ad-YAP–infected human islets (Figure 2, D–G), suggesting that increased FOXM1 is a crucial contributor of β cell proliferation in YAP-reconstituted β cells. Together, our

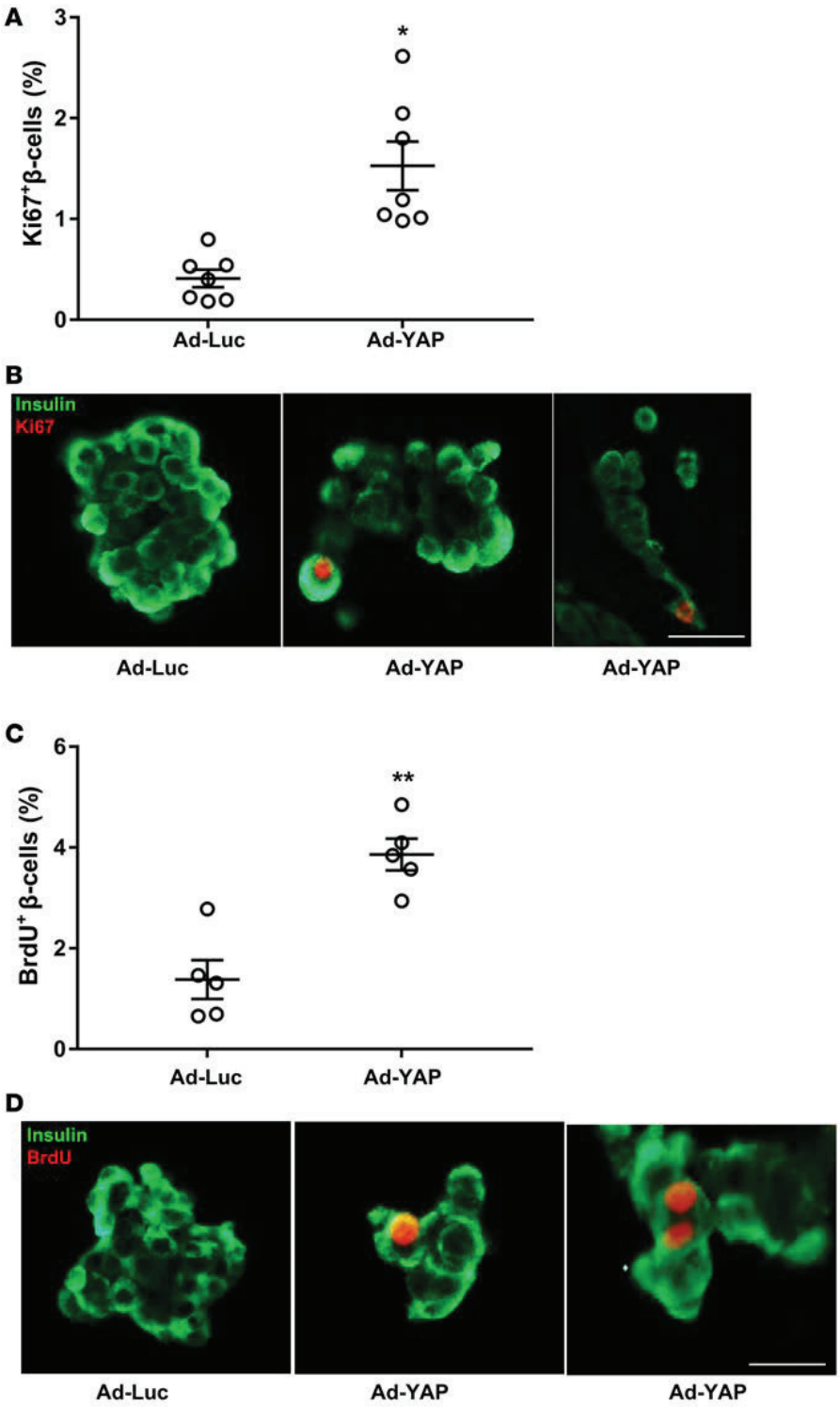

Figure 1. YAP overexpression promotes human β cell proliferation. (**A**–**D**) Human islets were infected with either control adenovirus expressing luciferase (Ad-Luc) or Ad-YAP for 2 days. β Cell proliferation was analyzed by double staining for Ki67 (**A** and **B**) or BrdU (**C** and **D**) (red) and insulin (green). Pooled data from 7 (**A**, $n = 7$) or 5 (**C**, $n = 5$) different human islet donors. Scale bars: 100 μm. Data are the mean ± SEM. $^*P < 0.001$, $^{**}P < 0.01$ for Ad-YAP– compared with Ad-Luc–infected islets by 2-tailed Student's t test.

data show that YAP robustly induced β cell proliferation in a FOXM1-dependent way.

Overexpression of constitutively active YAP does not alter β cell functional status. We then investigated whether YAP overexpression in human islets compromised their ability to secrete insulin in response to glucose in vitro. Glucose-stimulated insulin secretion (GSIS) assays in human islets infected with Ad-Luc or Ad-YAP showed that YAP-overexpressing islets maintained their insulin secretory capacity (Figure 3, A and B). To assess whether overexpression of YAP caused a loss of β cell identity or dedifferentiation, expression of functional genes including those encoding endocrine hormones (*INS* and *GCG*), key β cell transcription factors (*PDX1*, *NeuroD1*, *MafA*, *Nkx2.2*, *Nkx6.1*, and *Pax4*), as well as critical genes involved in glucose sensing (*GCK* and *Slc2a2*) were analyzed by RT-PCR in human islets and INS-1E cells and were fully preserved in Ad-YAP–infected or YAP-transfected cells (Figure 3, C and D). This result suggests full β cell identity and functionality upon YAP reexpression in islets. Despite the tendency of higher expression of some genes including *Nkx6.1* in Ad-YAP–infected islets, statistical analysis of pooled quantification data from 4 different human islet donors shows no significant differences for all analyzed genes except for *MafA* (Figure 3C; $P < 0.05$). *MafA* is a well-known regulator of insulin gene expression and is required for β cell maturation (37, 38), but its modest upregulation upon YAP overexpression did not alter insulin mRNA levels in our study. This is not surprising, as insulin gene expression is known to be regulated by a complex transcriptional network involving multiple transcription factors such as PDX1, NeuroD1, and MafA (39). Thus, the slight upregulation of one (*MafA*) — while 2 other critical transcription factors were unchanged (*NeuroD1* and *PDX1*) — was insufficient to enhance insulin mRNA transcription.

Overexpression of constitutively active YAP protects β cells from apoptosis under diabetic conditions. The important role of YAP-associated signaling in suppressing apoptosis led us to hypothesize that YAP activation would also protect β cells from apoptosis under diabetic conditions. INS-1E cells were transfected either

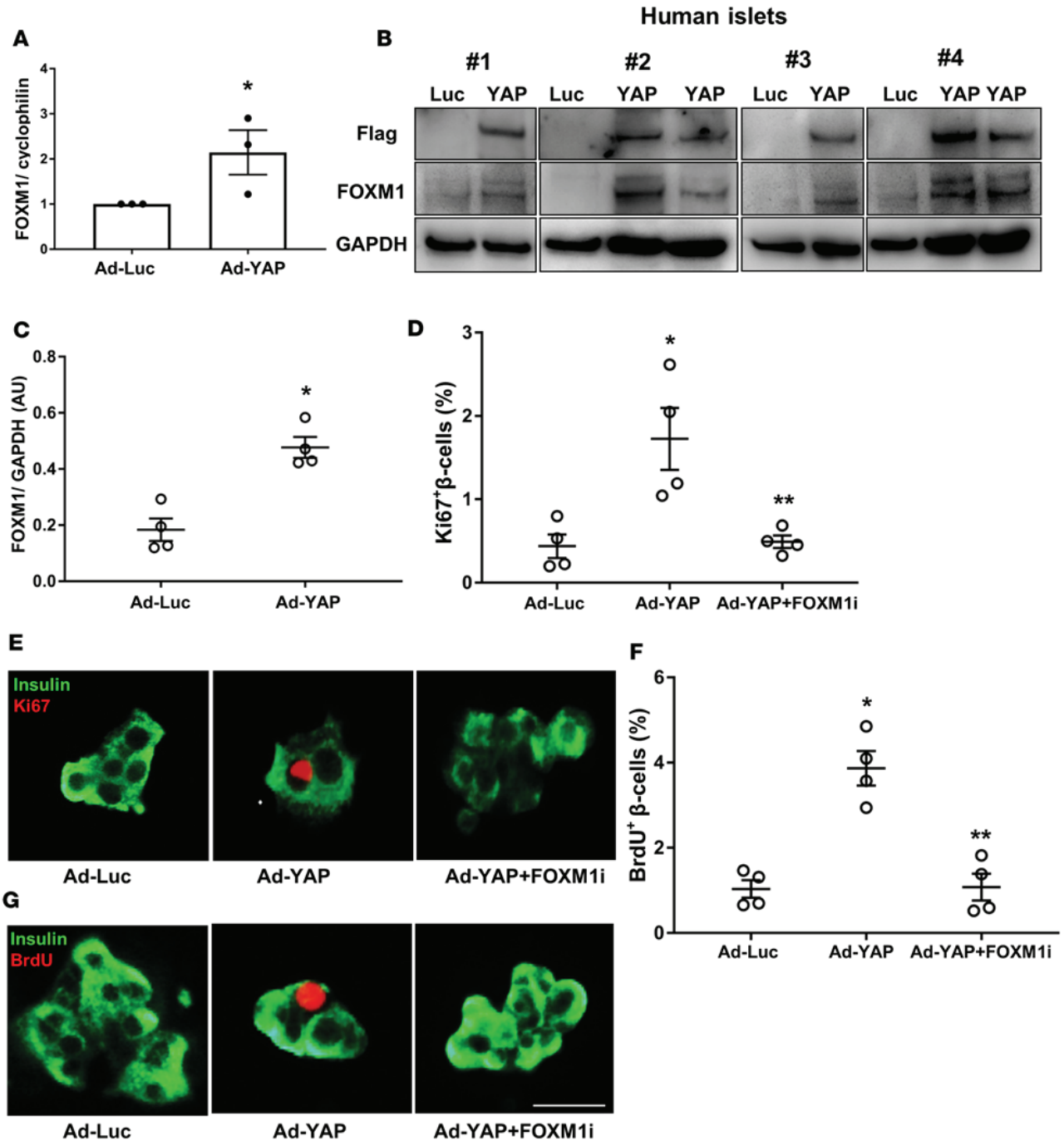

Figure 2. YAP-induced human β cell proliferation is FOXM1 dependent. (**A**) FOXM1 gene expression was analyzed by RT-PCR and levels normalized to cyclophilin and shown as change from control human islets infected with adenovirus expressing luciferase (Ad-Luc). Pooled data from 3 independent experiments from 3 different human islet donors (n = 3). (**B** and **C**) Western blots (**B**) and quantitative densitometry analysis (**C**) of FOXM1 protein level is shown. Western blots show results from 4 independent experiments from 4 different human islet donors (n = 4). (**D**–**G**) Human islets were infected with either control Ad-Luc or Ad-YAP and exposed to 10 μM FOXM1 inhibitor (thiostrepton; FOXM1i) for 2 days. β Cell proliferation was analyzed by double staining for Ki67 (**D** and **E**) or BrdU (**F** and **G**) (red) and insulin (green). (**D** and **F**) Pooled data from 4 independent experiments from 4 different human islet donors (n = 4). These 4 individual human islet data sets for Ad-Luc and Ad-YAP were also used for the quantification in Figure 1, A and C. Scale bar: 100 μm (applies to both **E** and **G**). Data are the mean ± SEM. *$P < 0.05$ for Ad-YAP compared with Ad-Luc–infected islets. **$P < 0.05$ for FOXM1i-treated Ad-YAP–infected compared with untreated Ad-YAP–infected islets. All 2-tailed Student's t test except **A** (1-tailed).

with YAP or control GFP plasmid and then exposed chronically to increased glucose concentrations (glucotoxicity), its combination with the free fatty acid palmitate (glucolipotoxicity), the mixture of proinflammatory cytokines interleukin-1β (IL-1β) and interferon γ (IFN-γ; cytokine toxicity), as well as acute exposure to hydrogen peroxide (H_2O_2, an in vitro model of oxidative stress). Overexpression of YAP markedly reduced glucose-, glucose/palmitate-, cytokine-, as well as H_2O_2-induced apoptosis as represented by decreased caspase-3 and PARP cleavage (Figure 4, A–F). Glucotoxicity and glucolipotoxicity as well as proinflammatory cytokines reduced the level of transfected YAP, which inversely correlated with caspase-3 and PARP cleavage (Figure 4, A and C). In order to further corroborate the antiapoptotic impact of reexpressed YAP in diabetes, we used an in vitro model of human islets exposed to a diabetogenic milieu of the proinflammatory cytokines or glucolipotoxicity. YAP was overexpressed by adenovirus-mediated infection of human islets isolated from nondiabetic organ donors. Apoptosis triggered by proinflammatory cytokines as well as by the mixture of high glucose/palmitate was counteracted by YAP overexpression in isolated human islets, demonstrated independently by 4 human islet isolations (representative Figures shown from 3 isolations; Figure 5, A and C). Our data show that YAP overexpression significantly blocked caspase-3 activation in human islets under diabetic conditions (Figure 5, B and D). These data show that YAP functions as a prosurvival signal to ameliorate the proapoptotic effect of diabetic conditions in the β cells.

The antiapoptotic effect of YAP is mediated by redox proteins Trx1/2. Since oxidative stress is an underlying mechanism of β cell destruction in diabetes (40–42) and ROS-associated signaling is regulated by YAP in other cell types (43, 44), we tested whether YAP overexpression would change expression of ROS-related genes. To identify YAP-regulated genes involved in the antioxidant system, we analyzed a panel of 25 ROS-related genes in the constitutively active YAP mutant–expressing or GFP-control–expressing INS-1E cells (data not shown). Our gene expression profiling revealed the components of the redox thioredoxin system including thioredoxin-1 (Trx1) and thioredoxin-2 (Trx2) as the most significantly upregulated genes in YAP-overexpressing β cells (Figure 6A). To further substantiate YAP-dependent regulation of Trx1/2, we measured the expression of Trx1/2 genes in isolated human islets. RT-PCR analysis showed that expression of Trx1 and Trx2 was higher in YAP-overexpressing human islets, compared with Ad-Luc controls (Figure 6B). Consistently, higher levels of Trx1 and Trx2 proteins were detected by Western blotting in YAP-overexpressing INS-1E cells (Figure 6, C and D) and human islets (Figure 6, E and F), confirming functional upregulation of Trx1/2 upon reexpression of YAP. Based on these observations, we sought to determine whether Trx1/2 is required for the prosurvival effect of YAP in β cells under diabetic conditions. Disruption of Trx1/2 redox equilibrium by an irreversible inhibitor of Trx1, 1-methylpropyl 2-imidazolyl disulfide (PX-12), or a chemical inhibitor of thioredoxin reductase (TrxR), auranofin, not only diminished the antiapoptotic effect of YAP but also highly exacerbated high-glucose-induced apoptosis as represented by upregulation of caspase-3 and PARP cleavage (Figure 6G). To further confirm that Trx1/2 mediates the effect of YAP on apoptosis inhibition, Trx1 and Trx2 were depleted by short interfering RNA (siRNA) in INS-1E cells. Loss of Trx1 or Trx2 potentiated glucose-induced apoptosis in the GFP control group as represented by cleavage of caspase-3 and PARP. The extent of induction (magnitude of amplification above basal high-glucose-treated and scrambled control siRNA–transfected cells, for both GFP- and YAP-transfected groups) was much lower in the YAP-transfected group; this correlates with remaining YAP present in Trx1- or Trx2-depleted cells (Figure 6H). Notably, Trx1/2 silencing reduced protein levels of exogenously expressed YAP; this correlated with massive induction of apoptosis and suggests the importance of cellular redox states for maintaining YAP protein levels (Figure 6H). Our data show a prosurvival mechanism of YAP action via Trx1/2 signaling and the functional significance of Trx1/2 as upstream effectors of YAP protein levels in the β cell.

Discussion

Diabetes results from an insufficient functional β cell mass. The identification of signaling molecules that foster β cell mass regeneration by simultaneous blockade of β cell apoptosis and augmentation of β cell proliferation would be highly important and most suitable for a β cell–directed therapy in diabetes. As regenerating β cells are similarly susceptible to apoptotic attack in general and even further under diabetes-relevant stressors such as inflammation and glucolipotoxicity, targets that induce β cell proliferation should ideally be combined with apoptosis-blocking strategies in order to maintain a functional β cell mass and thus to correct impaired glucose homeostasis. Here we show that the Hippo terminal effector YAP not only induced adult human β cell proliferation but also protected β cells from apoptosis induced by a diabetic milieu, suggesting

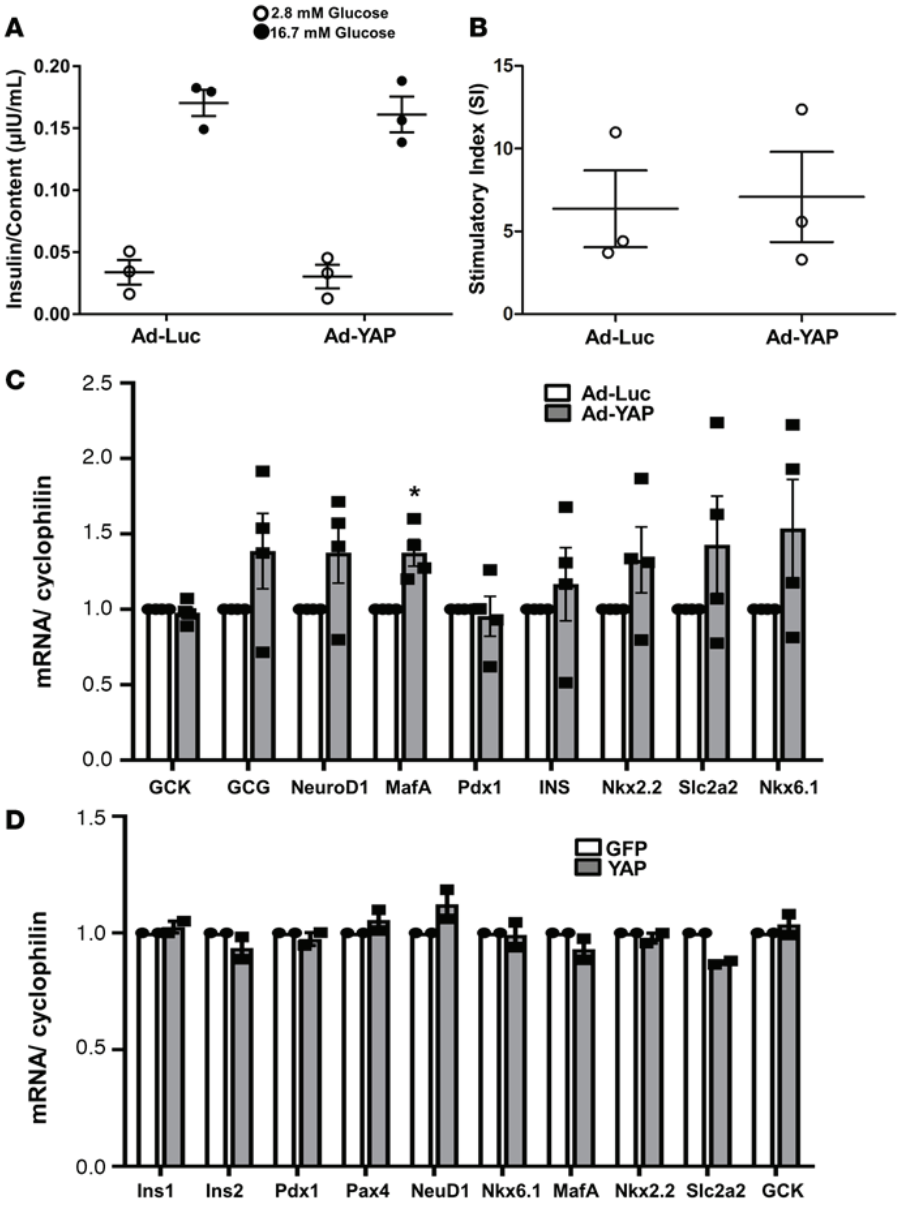

Figure 3. YAP overexpression does not alter β cell functional status. (**A**–**C**) Human islets were infected with either control adenovirus expressing luciferase (Ad-Luc) or Ad-YAP for 2 days. (**A**) Insulin secretion during a 1-hour incubation with 2.8 mM (basal) and 16.7 mM glucose (stimulated), normalized to insulin content. (**B**) The insulin stimulatory index denotes the ratio of secreted insulin during the 1-hour incubation with 16.7 mM and 2.8 mM glucose. (**A** and **B**) Pooled data from 3 independent experiments from 3 different human islet donors (n = 3). (**C**) RT-PCR for *GCK*, *GCG*, *NeuroD1*, *MafA*, *Pdx1*, insulin (*INS*), *Nkx2.2*, *Slc2a2*, and *Nkx6.1*. Pooled data from 4 independent experiments from 4 different human islet donors (n = 4). (**D**) INS-1E cells were transfected with either control GFP or YAP plasmid for 2 days. RT-PCR for *Ins1*, *Ins2*, *Pdx1*, *Pax4*, *NeuroD1*, *Nkx6.1*, *MafA*, *Nkx2.2*, *Slc2a2*, and *GCK*. Data are pooled data from 2 independent experiments (n = 2). (**C** and **D**) For analysis, we used the Applied Biosystems Step One Real-Time PCR system with TaqMan Fast Universal PCR Master Mix for TaqMan assays. Data are the mean ± SEM. *P < 0.05 for Ad-YAP–infected compared with Ad-Luc–infected islets by 2-tailed Student's t test.

that YAP is a potent factor involved in both β cell replication and survival.

Stimulating cell cycle reentry and proliferation of adult human β cells has proven to be very difficult and challenging (45–49). Processes that mediate the postnatal and age-dependent decline in proliferative and regenerative capacity of β cells remain largely unknown (50–53). Hypothetically, the loss of YAP signals right at the onset of pancreatic neurogenin-3 (Ngn3) expression and development of the endocrine pancreas (54) would reduce the ability of adult β cells to reenter the cell cycle and thus limit the regenerative potential of adult β cells. Overexpression of the YAP canonical target gene encoding connective tissue growth factor (CTGF) in a β cell regeneration model of partial pancreatectomy or treatment of human islets with recombinant human CTGF induces β cell proliferation (55), demonstrating that a YAP target acts directly on islets to promote β cell replication. Recent research has provided further insights into the importance of YAP in tissue regeneration. A functional consequence of the experimental elevation of YAP activity is the entry of nondividing or hardly dividing cells into the cell cycle, seen in both liver (56, 57) and heart (31, 58, 59). Together, these lines of evidence show that YAP transcriptional activity is enhanced and required for initiating cell proliferation and for complete tissue recovery in response to different types of injury. Hence, transient elevation of YAP activity might be useful for β cells that normally do not undergo proliferation and for augmenting the regenerative capability of damaged β cells in the context of diabetes.

Functional interaction of YAP with many proliferation-associated signaling networks contributes to its growth-promoting activity and is related to multiple physiological processes such as the regulation of cell cycle and proliferation (19–24, 32). YAP-dependent human β cell proliferation was accompanied and regulated by increased expression of transcription factor FOXM1. FOXM1 is a master regulator of cellular proliferation by controlling G1/S transition, S-phase progression, as well as G2/M transition.

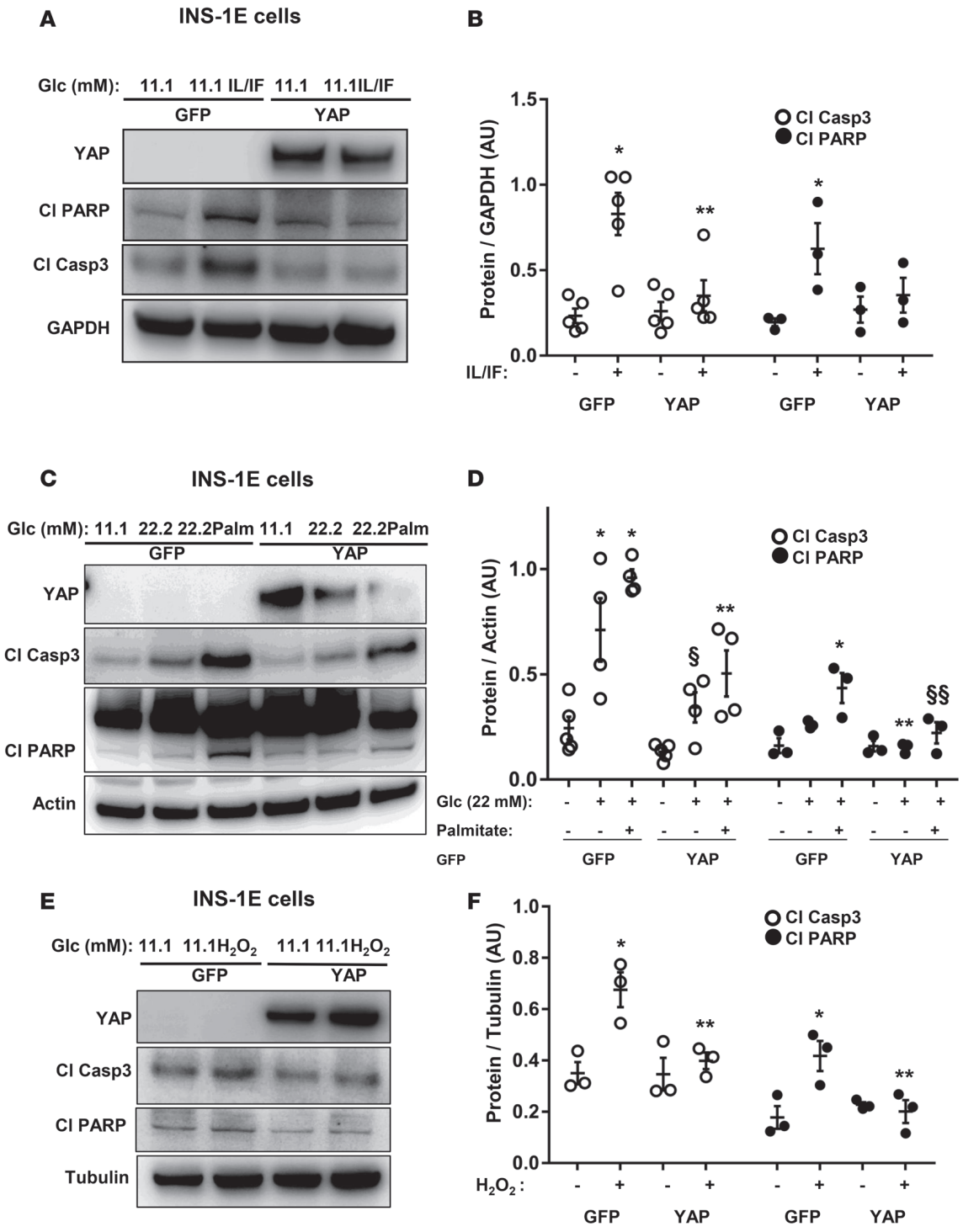

Figure 4. YAP overexpression protects β cells from apoptosis. (**A**–**F**) INS-1E cells were transfected with either control GFP or YAP plasmid and treated with (**A** and **B**) the proinflammatory cytokines recombinant human IL-1β (2 ng/ml) and 1,000 U/ml IFN-γ (IL/IF); or (**C** and **D**) 22.2 mM glucose (Glc) or the mixture of 22.2 mM glucose and 0.5 mM palmitate (22.2Palm) for 2 days; or (**E** and **F**) 25 μM H_2O_2 for 5 hours. Representative Western blots (**A**, **C**, and **E**) and quantitative densitometry analysis (**B**, **D**, and **F**) of cleaved caspase-3 (Cl Casp3) and cleaved PARP (Cl PARP) protein levels are shown. (**B**) Cl Casp3 (n = 5) and Cl PARP (n = 3). (**D**) Cl Casp3 (n = 4) and Cl PARP (n = 3). (**F**) Cl Casp3 (n = 3) and Cl PARP (n = 3). Data are the mean ± SEM. *P < 0.05 for treated GFP-transfected compared with untreated GFP-transfected condition. **P < 0.05 for treated YAP-transfected compared with treated GFP-transfected conditions. §P = 0.06 for 22.2 mM glucose–treated YAP-transfected compared with 22.2 mM glucose–treated GFP-transfected condition. §§P = 0.07 for 22.2Palm-treated YAP-transfected compared with 22.2Palm-treated GFP-transfected condition. All by 2-tailed Student's t test.

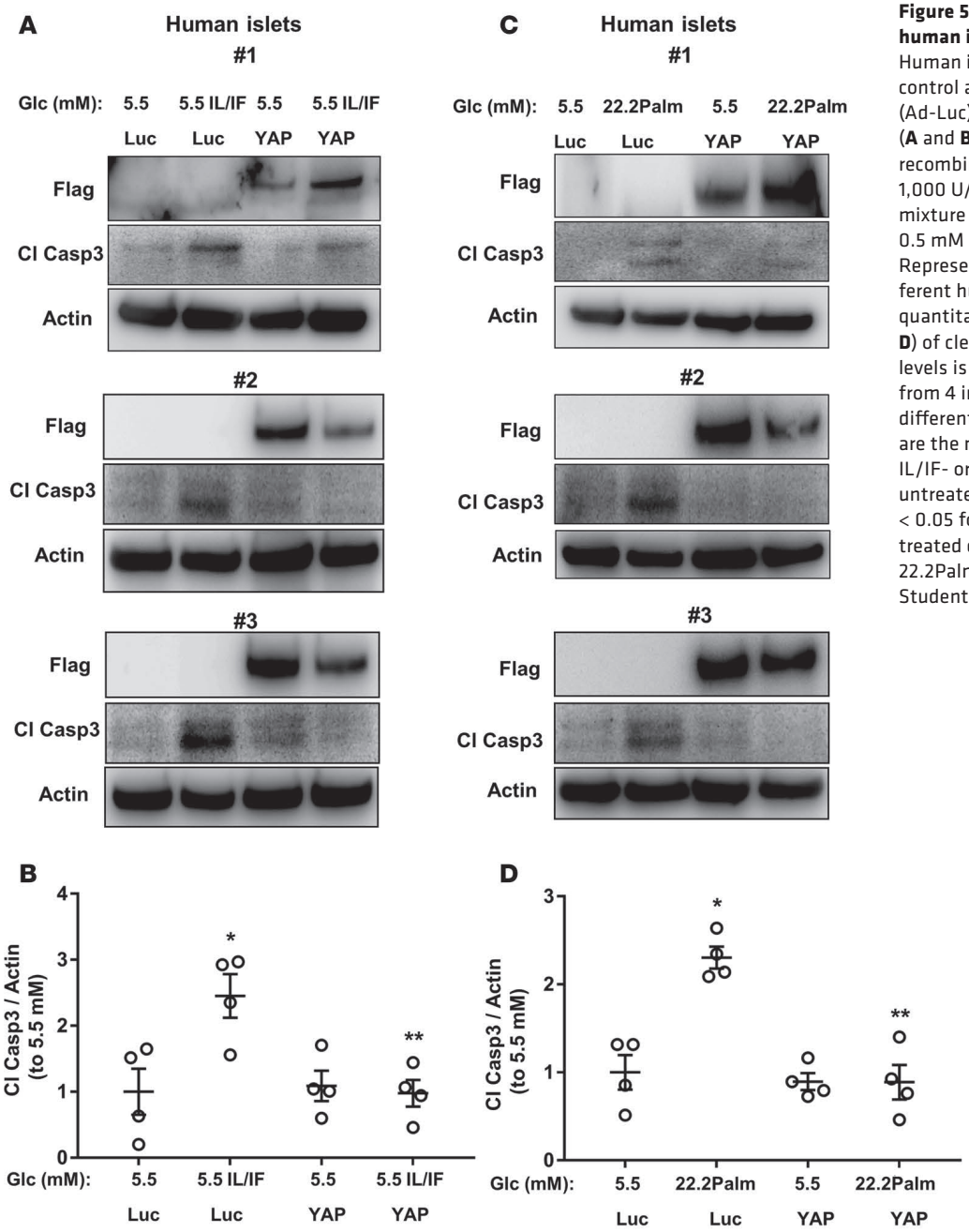

Figure 5. YAP overexpression protects human islets from apoptosis. (**A**–**D**) Human islets were infected with either control adenovirus expressing luciferase (Ad-Luc) or Ad-YAP and then treated with (**A** and **B**) the proinflammatory cytokines recombinant human IL-1β (2 ng/ml) and 1,000 U/ml IFN-γ (IL/IF) or (**C** and **D**) the mixture of 22.2 mM glucose (Glc) and 0.5 mM palmitate (22.2Palm) for 3 days. Representative Western blots of 3 different human islet donors (**A** and **C**) and quantitative densitometry analysis (**B** and **D**) of cleaved caspase-3 (Cl Casp3) protein levels is shown. (**B** and **D**) Pooled data from 4 independent experiments from 4 different human islet donors (n = 4). Data are the mean ± SEM. *P < 0.05 for Ad-Luc IL/IF- or 22.2Palm-treated compared with untreated Ad-Luc control condition. **P < 0.05 for Ad-YAP IL/IF- or 22.2Palm-treated compared with Ad-Luc IL/IF- or 22.2Palm-treated condition. All 2-tailed Student's t test.

Gannon's lab pioneered establishing the critical role of FOXM1 in postnatal β cell proliferation and mass expansion under conditions of increased β cell stress and demand in vivo (60, 61). Mice with pancreas-specific deletion of FOXM1 showed progressive β cell loss and overt diabetes (62). Conversely, FOXM1 overexpression alone is sufficient to induce β cell proliferation in mouse and human primary islets, highlighting the central role of FOXM1 in β cell proliferation (34). Consistent with the YAP-dependent FOXM1 upregulation in our study, Mizuno et al. reported that the YAP-TEAD complex directly binds to the FOXM1 promoter and induces transcriptional upregulation of FOXM1 in a cancer model of malignant mesothelioma (32). As YAP is a potential oncoprotein, interventions to increase β cell proliferation raise concerns about the possibility of neoplastic growth. Thus, YAP-based therapeutic approaches to restore lost or damaged β cells should be combined with strategies designed to limit YAP overactivation and uncontrolled cell proliferation. The acute and targeted manipulation of YAP may keep its levels physiological, tightly controlled, and ideally sufficient for modest β cell proliferation, mass expansion, and restoration of glucose homeostasis.

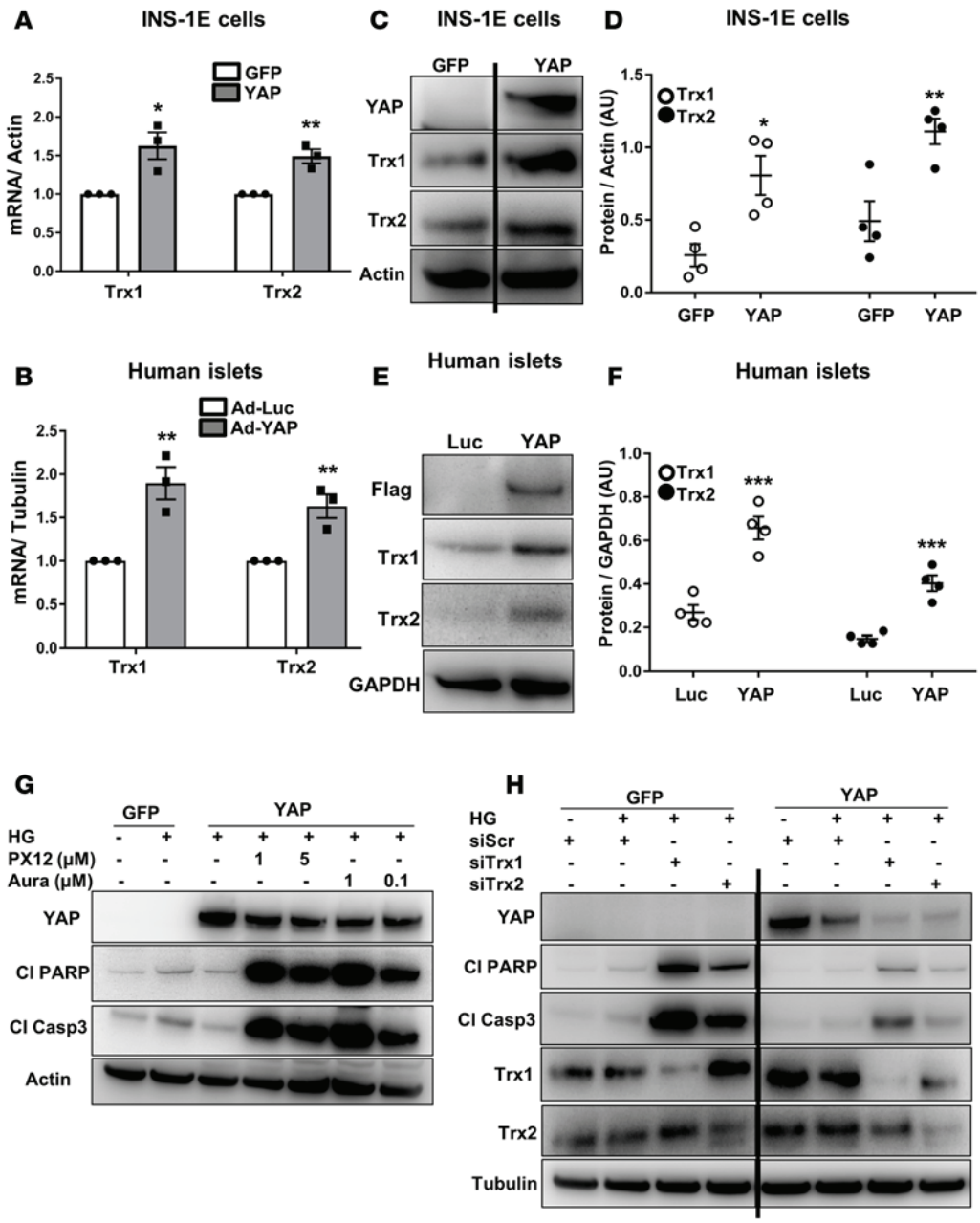

Figure 6. Antiapoptotic effect of YAP is mediated by redox proteins Trx1/2. (**A**, **C**, and **D**) INS-1E cells were transfected with either control GFP or YAP plasmid for 2 days. (**A**) Trx1 and Trx2 gene expression was analyzed by RT-PCR and levels normalized to actin and shown as change from GFP-transfected cells. Pooled data from 3 independent experiments (n = 3). (**C** and **D**) Representative Western blots (**C**) and quantitative densitometry analysis (**D**) of Trx1 and Trx2 protein levels are shown. Both lanes were run in the same gel but were noncontiguous. Pooled data from 4 independent experiments (n = 4). (**B**, **E**, and **F**) Human islets were infected with either control adenovirus expressing luciferase (Ad-Luc) or Ad-YAP for 2 days. (**B**) Trx1 and Trx2 gene expression was analyzed by RT-PCR and levels normalized to tubulin and shown as change from Ad-Luc–infected human islets. Pooled data from 3 independent experiments from 3 human islet donors (n = 3). (**E** and **F**) Representative Western blots (**E**) and quantitative densitometry analysis (**F**) of Trx1 and Trx2 protein levels are shown. Pooled data from 4 independent experiments from 4 different human islet donors (n = 4). Flag and GAPDH blots in **E** are reused from human islet number 3 in Figure 2B. (**G**) INS-1E cells were transfected with either control GFP or YAP plasmids and treated with 22.2 mM glucose (HG) for 2 days. PX-12 (Trx1 inhibitor) and auranofin (thioredoxin reductase inhibitor) were added for the last 24 hours. (**H**) INS-1E cells were transfected with either control GFP or YAP and scrambled control siRNA (siScr) or Trx1 or Trx2 siRNA and treated with 22.2 mM glucose (HG) for 2 days. All lanes were run in the same gel but were noncontiguous. Western blots show representative results from 3 independent experiments (n = 3). Data are the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for YAP compared with control GFP- or Luc- transfected/infected conditions by 2-tailed Student's t test.

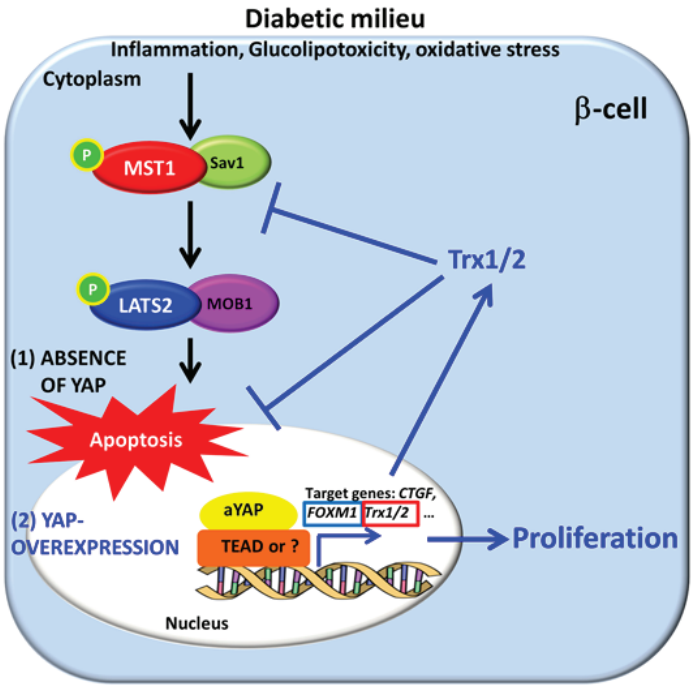

Figure 7. A model for how β cell proliferation and apoptosis are regulated by exogenously introduced YAP. Various diabetic stimuli lead to activation of central Hippo signaling kinases MST1/LATS2. (1) In the absence of YAP, this activation results in collective transcriptional and posttranscriptional events leading to β cell apoptosis and impaired proliferation (black arrows). (2) Reexpression of the constitutively active form of YAP, which is absent in the mature β cell and functionally insensitive to the inhibitory action of MST1/LATS2 signaling, induces expression of a plethora of genes such as Trx1/2 and FOXM1 with antiapoptotic and proproliferative output, which protects β cells from diabetogenic attack (blue arrows). YAP-induced Trx1/2 upregulation and Trx1/2-mediated YAP regulation (through MST1) forms a complex regulatory signaling loop, which controls redox state and cell survival.

Our data show that overexpression of the constitutively active form of YAP effectively diminished β cell death triggered by multiple diabetic conditions in vitro including proinflammatory cytokines, gluco- and lipotoxicity, and oxidative stress. The prosurvival effect of YAP reexpression in stressed β cells induced by different diabetic stimuli raises the possibility that the antiapoptotic outcome of YAP comes from coordinating and regulating a common antiapoptotic gene expression program that promotes β cell protection. By gene expression profiling and subsequent protein analysis, we demonstrate that YAP induced profound upregulation of Trx system components in β cells. In line with our data, 2 independent studies showed YAP-induced upregulation of Trx components either through liver-specific deletion of MST1/2 and subsequent YAP activation or direct activation of YAP (44, 63). The Trx system consists of NADPH, TrxR, and Trx and is a redox-sensitive signaling system that protects cells from oxidative stress. It has emerged as a key element linking redox regulation to the pathogenesis of several diseases (64). Oxidative stress has been recognized as one of the common underlying mechanisms of β cell failure in the pathogenesis of both types of diabetes (40–42). In vivo β cell–specific overexpression of Trx1 protects mice from development of diabetes in both T1D and T2D rodent models, demonstrating the efficacy of Trx1 to halt the progressive β cell demise (65, 66). Trx1 also functions as the molecular sensor for the redox-dependent regulation of MST1 activity; Trx1 inhibits stress-induced MST1 activation by directly compromising MST1 homodimerization and autophosphorylation (67). MST1 hyperactivity is a major underlying mechanism of β cell apoptosis and the consequent decrease in β cell mass in the diabetic state (28). Thus, Trx1 might function as a YAP-dependent molecular signal to turn off the prodiabetic milieu–induced MST1 activation and subsequent apoptosis as part of compensatory negative-feedback loop within the Hippo signaling. Further detailed investigation is required to support such a possibility. As highlighted, YAP overexpression induced Trx1/2 at mRNA and protein levels in both human islets and INS-1E cells in control (Figure 6, A–F) and diabetic states (Figure 6H). On the other hand, Trx1/2 silencing itself reduced exogenously expressed YAP protein (Figure 6H); this suggests that the Trx1/2 system — or the cellular redox state in general — controls the level of YAP. Thus, shifting the cellular redox environment to pro-oxidant states by loss of Trx1 or Trx2 promotes YAP degradation and reverses the prosurvival effect of YAP. Together, YAP-induced Trx1/2 upregulation and Trx1/2-mediated YAP regulation by MST1 (as speculated above) form complex regulatory signaling loops (see scheme in Figure 7), which control redox state and cell survival.

In full agreement with our study, George et al. (54) also reported the robust induction of human β cell proliferation by YAP activation with preservation of insulin-secretory function. In this study, Ngn3-dependent specification during development of the endocrine pancreas is sufficient for loss of YAP and decreased proliferation. Also, mechanistic (or mammalian) target of rapamycin (mTOR) signaling was proposed as an underlying mechanism of YAP-induced β cell proliferation (54). We have not directly assessed whether mTOR activity is regulated in YAP-overexpressing human islets, but FOXM1 might function as a part of mTOR-associated signaling in YAP-hyperactivated cells. This link between mTOR and FOXM1 was previously identified for epidermal growth factor receptor (EGFR) signaling–mediated β cell proliferation in response to overnutrition (68). Consistent with George et al. (54), most of the BrdU- and insulin-positive or Ki67- and insulin-positive cells were YAP positive, suggesting mostly a

cell-autonomous proproliferative action of YAP. However, a non–cell autonomous effect of YAP overexpression cannot entirely be ruled out. For example, CTGF, an established canonical YAP target gene, is highly upregulated upon YAP overexpression in islets, as shown by George et al. (54) as well as in our study in human islets (data not shown). CTGF is a secreted growth factor that is capable of promoting human β cell proliferation and β cell regeneration in a model of partial pancreatectomy (55). Thus, highly produced CTGF and/or other uncharacterized secreted proteins upon YAP reconstitution may trigger proliferation in YAP-negative cells by a paracrine mechanism. Further studies are required to reveal such a possibility.

Conclusion. Insufficient and dysfunctional β cell mass is a central feature of both types of diabetes. Thus, strategies aiming to regenerate pancreatic β cell mass by blocking β cell apoptosis as well as promoting proliferation are urgently needed. Considering the remarkable antiapoptotic and proproliferative output of YAP reexpression in β cells, manipulation of YAP may serve as a therapeutic tool for restoring functional β cell mass in diabetes. To warrant this, further mechanistic studies as well as the in vivo preclinical assessment of efficacy and side effects of YAP overexpression in β cells are required. Here, owing to its oncogenic potential, YAP activity must be strictly regulated.

Methods

Cell culture, treatment, and islet isolation. Human islets were isolated from pancreases of healthy organ donors at the University of Chicago and at ProdoLabs and cultured on extracellular matrix–coated (ECM-coated) dishes (Novamed) as described previously (69). The clonal rat β cell line INS-1E was provided by Claes Wollheim, the University of Geneva, Geneva, Switzerland and the University of Lund, Lund, Sweden. Human islets were cultured in complete CMRL-1066 (Invitrogen) medium at 5.5 mM glucose and INS-1E cells in complete RPMI-1640 (Invitrogen) medium at 11.1 mM glucose as described previously (28). Islets and INS-1E were exposed to complex diabetogenic conditions (28). In some experiments, cells were additionally cultured with 10 μM FOXM1 inhibitor thiostrepton (Calbiochem), 1–5 μM selective Trx1/2 inhibitor PX-12, and 0.1–1 μM TrxR inhibitor auranofin (both Cayman Chemical). BrdU was added to the culture media at 10 μM for 2 days to label proliferating cells.

Transfections. To knock down Trx1 and Trx2 in INS-1E cells, SMARTpool technology from Dharmacon was used. A mix of ON-TARGETplus siRNAs directed against the following sequences: in rat *Txn1*, GCAAAAUGAUCAAGCCCUU, GGAUGUUGCUGCAGACUGU, AGCUUGUGGUAGUGGACUU, CCUUGAAGUAGGACGUGGAU; and rat *Txn2*, GAUCCUAGGACCUCGGUUA, UAGCCAAACAGCACGGGAA, CAAAGUGGACAUUGACGAU, GAAGCUAAUUGGCUGACAA (100 nM, Dharmacon) was transiently transfected into INS-1E cells as described previously (28). GFP and YAP (pCMV-flag YAP^{S127A}, a gift from Kunliang Guan [Addgene plasmid number 27370]) plasmids were used to overexpress these proteins in INS-1E cells.

Glucose-stimulated insulin secretion. Glucose-stimulated insulin secretion in human islets was performed as described previously (28).

Immunohistochemistry. Human islets cultured on ECM dishes were fixed, permeabilized, blocked, and incubated overnight at 4°C with anti–human Ki67 (catalog 08-1192; Invitrogen), mouse anti-BrdU (catalog NA61; Calbiochem), rabbit anti-Flag (catalog F7425; Sigma-Aldrich) and anti-insulin (catalog A0546; Dako) antibodies followed by fluorescein isothiocyanate (FITC)-, Cy3-, or AMCA-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Slides were mounted with Vectashield with 4′,6-diamidino-2-phenylindole (DAPI; Vector Labs). Fluorescence was analyzed using a Nikon MEA53200 microscope and images were acquired using NIS-Elements software (Nikon).

Western blot analysis. Western blotting was performed as described previously (28). Membranes were incubated overnight at 4°C with rabbit anti–cleaved caspase-3 (catalog 9664), rabbit anti-Trx1 (catalog 2429), rabbit anti-Trx2 (catalog 14907), rabbit anti-YAP (catalog 4912), rabbit anti-PARP (catalog 9532), rabbit anti–cleaved PARP (rat specific, catalog 9545), rabbit anti-tubulin (catalog 2146), rabbit anti-GAPDH (catalog 2118), rabbit anti–β-actin (catalog 4967) (all Cell Signaling Technology), rabbit anti-FOXM1 (catalog ab55006; Abcam), and rabbit anti-Flag (catalog F7425; Sigma-Aldrich), followed by horseradish peroxidase–linked anti–rabbit IgG (Jackson ImmunoResearch Laboratories). Membranes were developed using a chemiluminescence assay system (Pierce) and analyzed using DocIT LS image acquisition 6.6a (UVP BioImaging Systems). Densitometric analysis of the immunoblots was performed using Vision Works LS Image Acquisition and Analysis software Version 6.8 (UVP BioImaging Systems).

RNA extraction and RT-PCR analysis. Total RNA was isolated from cultured human islets and INS-1E cells using TriFast (PEQLAB Biotechnologie), and RT-PCR performed as described previously (28). For analysis, we used the StepOne Real-Time PCR system with TaqMan Fast Universal PCR Master Mix for TaqMan assays (both Applied Biosystems). TaqMan Gene Expression Assays were used for *PDX1* (Hs00426216_m1), *SLC2A2* (Hs01096905_m1), *GCK* (Hs01564555_m1), *INS* (Hs02741908_m1), *GCG* (Hs01031536_s1), *Nkx2.2* (Hs00159616_m1), *MAFA* (Hs01651425_s1), *Nkx6.1* (Hs00232555_m1), *NeuroD1* (HS01922995_s1), *FoxM1* (Hs01073586_m1), and *PPIA* (Hs99999904_m1) for human and *PDX1* (Rn00755591_m1), *SLC2A2* (Rn00563565_m1), *GCK* (Rn00688285_m1), *INS1* (Rn02121433_g1), *INS2* (Rn01774648_g1), *Nkx2.2* (Rn04244749_m1), *Nkx6.1* (Rn01450076_m1), *NeuroD1* (Rn00824571_s1), *MafA* (Rn00845206_s1), *Pax4* (Rn00582529_m1) and *PPIA* (Rn00690933_m1) for rat or the SYBR Green Real-Time PCR Kit (Applied Biosystems) for mRNA expression of ROS-regulated genes with actin or tubulin as housekeeping controls for all experiments. The following primers were used: rat *Txn1* Fw 5′-GTCAAATGCATGCCGACCTT-3′ and Rev 5′-AGTGGCTTC-GAGCTTTTCCTT-3′; rat *Txn2* Fw 5′-AAGCCTCCTCAAGGTGTGTG-3′ and Rev 5′-AGACCAC-CAGCATTGTACGG-3′; human *TXN1* Fw 5′-GATGTGGATGACTGTCAGGATGT-3′ and Rev 5′-TCACCCACCTTTTGTCCCTTC-3′; human *TXN2* Fw 5′-AGAGAAGATGGTGGCCAAGC -3′ and Rev 5′-CTCAATGGCGAGGTCTGTGT-3′.

Adenovirus infection. Isolated human islets were infected with Ad-Luc as a control (provided by Allan E. Karlsen, Novo Nordisk A/S, Denmark) or active YAP (Ad-YAP^{S127A} with an N-terminal triple FLAG epitope tag, provided by William Pu, Harvard University, Cambridge, Massachusetts, USA) at a multiplicity of infection (MOI) of 100 for 4 hours. Adenovirus was washed off with PBS and replaced by fresh complete medium. Staining, GSIS, and RNA and protein isolations were performed at 48, 72, or 96 hours after infection.

Statistics. Samples (human islets) were evaluated in a randomized manner by 2 investigators (TY, ZA) who were blinded to the treatment conditions. All values were expressed as means ± SEM with the number of independent individual experiments presented in the figure legends. The different groups were compared by 2-tailed Student's *t* test unless otherwise mentioned. A *P* value less than 0.05 was considered statistically significant.

Study approval. Ethical approval for the use of human islets had been granted by the Ethics Committee of the University of Bremen.

Author contributions

AA conceived the project. TY and AA designed the project. TY, SR, ZA, BL, SA, KG, and AA preformed experiments and analyzed data. JO contributed new reagents or analytic tools. TY, KM, and AA wrote the paper. AA and KM supervised the project.

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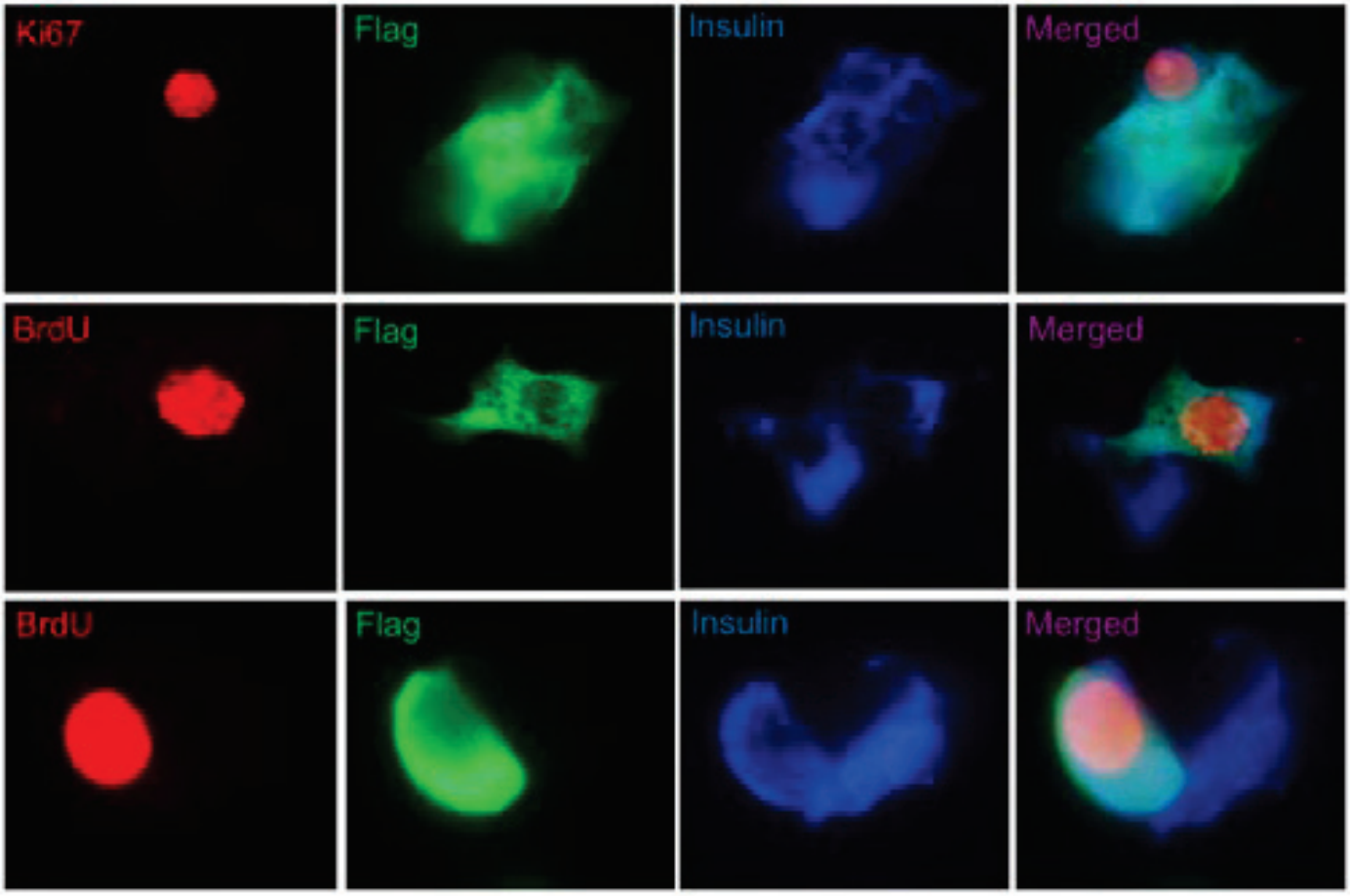

Supplementary Figure 1. Triple staining of YAP (Flag-tag), insulin and Ki67 or BrdU. Human islets were transfected with a Flag-YAP for 2 days. β-cell proliferation was analyzed by triple staining for Ki67 or BrdU (red), Flag (green) and insulin (blue).

Manuscript III

2.3 Reciprocal regulation of mTOR complexes in human type 2 diabetic pancreatic islets

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Designed and performed experiments (contributed to Figures 1, 4, 5 and 6), and analyzed data.

Reciprocal regulation of mTOR complexes in human type 2 diabetic pancreatic islets

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Running Title

mTORC1 activity in type 2 diabetic islets

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Abstract

Aims/hypothesis: Mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of nutritional status at the cellular and organismic level. While mTORC1 mediates beta cell growth and expansion, its hyper-activation has been observed in pancreatic islets from animal models of type 2 diabetes and leads to beta cell loss. We sought to determine whether such mTORC1 activation also occurs in human type 2 diabetic or metabolically stressed human islets and whether mTORC1 blockade can restore beta cell function of diabetic islets.

Methods: Human islets isolated from non-diabetic controls and individuals with type 2 diabetes as well as treated human islets and INS-1E cells with increased glucose (22.2 mmol/l) were analyzed for mTORC1/2 activity by Western blot analysis of phosphorylation of mTORC1 down-stream targets S6K1, S6 and 4E-BP1 and mTORC2 down-stream targets AKT and NDRG1. mTORC1/2 complexes' integrity was assessed by immunoprecipitation and subsequent Western blot analysis. Cell-type specific expression of activated mTORC1 in human islets was examined by immunostaining of pS6 (Ser 235/236) in human islet sections. Beta cell function was measured by glucose-stimulated insulin secretion (GSIS).

Results: While mTORC2 signalling was diminished, mTORC1 activity was markedly increased in type 2 diabetic islets and in islets and beta cells exposed to increased glucose concentrations. Under such increased glucose conditions in metabolically stressed human islets, we identified a reciprocal regulation of different mTOR complexes with functional upregulation of mTORC1 and downregulation of mTORC2. pS6 immunostaining showed beta cell-specific up-regulation of mTORC1 in islets isolated from patients with type 2 diabetes. Inhibition of mTORC1-S6K1 signaling improved glucose-induced insulin secretion and restored mTORC2 activity in type 2 diabetic islets as well as in isolated diabetic islets from diabetic db/db and high-fat/ high sucrose diet (HFD) fed mice.

Conclusions/interpretation: Our data show the aberrant mTORC1 activity in islets from patients with type 2 diabetes, in human islets cultured under diabetes-associated increased glucose conditions and in diabetic mouse islets. This suggests elevated mTORC1 activation

as striking pathogenic hallmark of type 2 diabetic islets contributing to impaired beta cell function and survival in the presence of metabolic stress.

Keywords
Beta cells, glucose, human islets, mTORC1, mTORC2, nutrients, Type 2 Diabetes

Abbreviations:

Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1, 4E-BP1

Glucose-stimulated insulin secretion, GSIS

Mechanistic target of rapamycin, mTOR

mTOR complex-1, mTORC1

mTOR complex-2, mTORC2

NEFA, non-esterified fatty acid

N-Myc downstream regulated 1, NDRG1

Ribosomal protein S6 kinase 1, S6K1

High-fat/ high sucrose diet, HFD

Introduction

The pancreatic beta cells play a central role in controlling glucose homeostasis in response to metabolic fluctuations. To do this, beta cells must coordinate multiple cellular metabolic processes in order to adapt their insulin secretory responses with nutrient availability. Type 2 diabetes manifests itself by a progressive decline in beta cell functional adaptation and ultimate loss of beta cell mass by apoptosis [1]. The compensatory insulin response by pancreatic beta cells is a key rate-limiting step determining whether at-risk obese individuals develop diabetes or not. Nutrient overload such as chronically increased glucose concentrations and non-esterified fatty acids (NEFAs) have been proposed as main underlying pathological factors leading to beta cell deterioration during progression of type 2 diabetes [2]. Identifying the signaling mechanisms by which nutrient-overload leads to impaired insulin action and beta cell death is instrumental for better understanding of the molecular changes underlying functional beta cell mass loss and development of therapeutic strategies for the treatment of type 2 diabetes.

The mechanistic target of rapamycin (mTOR) is a master regulator of diverse cellular functions such as metabolism, proliferation and survival by forming at least two functionally distinct complexes, mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2). While mTORC1 phosphorylates various substrates such as eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), S6 kinase 1 (S6K1) and SREBP to promote anabolic processes, mTORC2 phosphorylates AGC kinases including AKT and SGK1 to enhance cell proliferation and survival exerting their biological functions. mTORC1 is a key nutrient sensor by integrating diverse extra- and intra-cellular cues to down-stream signaling pathways in response to growth factors, stress, nutrient availability and other stimuli [3]. Considering the importance of metabolic control and mTORC1's role as key sensor and regulator of cellular energy, it is not surprising that this signaling is dysregulated in a variety of disease states such as cancer and type 2 diabetes [3, 4]. mTORC1 activity is highly up-regulated in the liver, fat, muscle and pancreatic islets of obese and high-fat-fed rodents [5-8]; this correlates with elevated circulating levels of insulin, pro-inflammatory cytokines, and nutrients such as glucose and NEFAs and may represent a common consequential mechanism triggered by these driving forces in obesity. mTORC1 activity is constitutively elevated under conditions of

nutrient satiation in the rodent beta cell line in vitro [9] and in the pancreatic islets of type 2 diabetic mice [8], We therefore asked in the present study whether mTORC1 activity is altered in human isolated islets under conditions of metabolic stress and diabetes and whether acute inhibition of mTORC1 signaling can improve beta cell function.

Methods

Human islet isolation, culture and treatment

Human islets were isolated from pancreases of non-diabetic organ donors and from patients with type 2 diabetes at the University of Illinois at Chicago, Geneva University, Leiden University, Lille University and at ProdoLabs (Table 1) and cultured on extracellular matrix (ECM) coated dishes (Novamed, Jerusalem, Israel) as described previously [10]. The clonal rat beta-cell line INS-1E was kindly provided by Dr. Claes Wollheim, Geneva & Lund University. Human islets were cultured in complete CMRL-1066 (Invitrogen) medium at 5.5 mmol/l glucose and INS-1E cells at complete RPMI-1640 medium at 11.1 mmol/l glucose and were exposed chronically to increased glucose at 22.2 mmol/l. In some experiments, human islets were additionally cultured with 10 µmol/l S6K1 selective inhibitor PF-4708671 (Calbiochem). Ethical approval for the use of human islets was granted by the Ethics Committee of the University of Bremen.

Animals, islet isolation and culture

For the high-fat/ high sucrose diet (HFD) experiments, C57BL/6J male mice originally obtained from Jackson Laboratory were fed a normal diet (ND, Harlan Teklad Rodent Diet 8604, containing 12.2, 57.6 and 30.2% calories from fat, carbohydrate and protein, respectively) or a high-fat, high-sucrose diet (HFD, Surwit Research Diets, New Brunswick, NJ, containing 58, 26 and 16% calories from fat, carbohydrate and protein, respectively [11]) for 17 weeks. Heterozygous leptin receptor deficient mice on the C57BLKS/J background ($Lepr^{db/+}$, $db/^{+}$) were purchased from Jackson Laboratory. By breeding of these mice, we obtained diabetic $Lepr^{db/db}$ (db/db) as well as non-diabetic heterozygous $Lepr^{db/+}$ (db/+) mice. After 17 weeks of HFD or at the age of 12 weeks (db/db), islets were isolated as described previously [10]. Pancreases were perifused with a Liberase TM (#05401119001, Roche, Mannheim, Germany) solution according to the manufacturer's instructions and digested at

37°C, followed by washing and handpicking. High purity islets were cultured overnight in RPMI 1640 medium containing 11.1 mM glucose, treated with or without S6K1 selective inhibitor PF-4708671 for 4 hours following protein isolation or glucose stimulated insulin secretion (GSIS). All animals were housed in a temperature-controlled room with a 12-h light-dark cycle and were allowed free access to food and water in agreement with US National Institutes of Health animal care guidelines and the German animal protection law and approved by the Bremen Senate.

Transfections

To knock down Raptor expression in human islets, SMARTpool technology from Dharmacon was used. A mix of ON-TARGETplus siRNAs directed against human *Raptor* sequences UGGCUAGUCUGUUUUCGAAA, CACGGAAGAUGUUCGACAA, AGAAGGGCAUUACGAGAUU and UGGAGAAGCGUGUCAGAUA (100 nmol/l, Dharmacon) was transiently transfected into human islets and efficiently reduced Raptor levels. An ON-TARGETplus nontargeting siRNA pool from Dharmacon served as control. An adapted improved protocol to achieve silencing in human islets was used as described previously [10]. Islets were partially dispersed with accutase (PAA) to break islets into smaller cell aggregates to increase transfection efficiency and cultured on ECM dishes for 1 day. Isolated islets were exposed to transfection Ca^{2+}-KRH medium (KCl 4.74 mmol/l, KH2PO4 1.19 mmol/l, MgCl2•6H2O 1.19 mmol/l, NaCl 119 mmol/l, CaCl2 2.54 mmol/l, NaHCO3 25 mmol/l and HEPES 10 mmol/l). After 1-h incubation, lipoplexes (Lipofectamine 2000, Invitrogen)/siRNA ratio 1:20 pmol) were added to transfect the islets cells. After an additional 6 hour incubation, CMRL-1066 medium containing 20% FCS and l-glutamine was added to the transfected islet cells.

Western Blot analysis

Western blotting was performed as described previously [10]. Membranes were incubated overnight at 4°C with rabbit anti-pS6 ribosomal protein (Ser 235/236, #4858), rabbit anti-p-p70S6K (Thr 389, #9234), rabbit anti-p4E-BP1 (Thr 37/46, #2855), rabbit anti-pAKT (Ser 473, #4058), rabbit anti-pNDRG1 (Thr 346, #5482), rabbit anti-mTOR (#2983), rabbit anti-raptor (#2280), rabbit anti-rictor (#2114), rabbit anti-GAPDH (#2118) and rabbit anti-β-actin (#4967) (all Cell Signaling Technology, CST) followed by

horseradish-peroxidase-linked anti-rabbit IgG (Jackson). All primary antibodies were used at 1:1000 dilution in Tris-buffered saline plus Tween-20 (TBS-T) containing 5% BSA. Membranes were developed using a chemiluminescence assay system (Pierce) and analyzed using DocIT®LS image acquisition 6.6a (UVP BioImaging Systems, Upland, CA, USA).

Co-Immunoprecipitation

Immunoprecipitation was performed as described previously [12]. Briefly, human islets were lysed in cold buffer containing 0.4% CHAPS, 50 mmol/l Tris-HCl [pH 7.4], 150 mmol/l NaCl, 10 mmol/l MgCl2, 50 mmol/l β-glycerophosphate supplemented with proteinase/phosphatase inhibitors for 30 min on ice. Lysates were centrifuged at 12,000 g for 15 min at 4°C prior to immunoprecipitation. This starting material before IP (input) was loaded on the western blot in order to compare the endogenous expression of the respective proteins. Immunoprecipitations were carried out by incubating 500 µg of total lysate with rabbit anti-mTOR (#2983, CST) (1:100) on a rotator at 4°C overnight. Immunocomplexes were then captured with Protein A Agarose Fast Flow (Millipore) by rotation at 4°C for 4 h. After five washes with cold lysis buffer, the immunoprecipitates were resuspended in sample buffer and separated by NuPAGE 4-12% Bis-Tris gels (Invitrogen).

Immunofluorescence

Isolated human islets were cultured overnight and fixed in Bouin's solution for 15 min before embedding in paraffin as previously described [13]. Human 4-µm sections were deparaffinized, rehydrated and incubated overnight at 4ºC with primary antibodies followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted with Vectashield with 4'6-diamidino-2-phenylindole (DAPI; Vector Labs). The following primary antibodies were used: rabbit anti-pS6 ribosomal protein (Ser 235/236, #4858, 1:100, CST) in combination with TSA (Invitrogen #T30955), guinea pig anti-insulin (#A0546, 1:100, Dako) and mouse anti-glucagon (#G2654, 1:100, Sigma). Fluorescence was analyzed using a Nikon MEA53200 (Nikon GmbH, Dusseldorf, Germany) microscope and images were acquired using NIS-Elements software (Nikon).

Glucose stimulated insulin secretion

For acute insulin release in response to glucose, primary human islets were washed and pre-incubated (30 min) in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mmol/l glucose and 0.5% BSA. KRB was then replaced by KRB 2.8 mmol/l glucose for 1 h (basal), followed by an additional 1 h in KRB 16.7 mmol/l glucose (stimulated). Total protein content was extracted with RIPA buffer. Insulin was determined using human insulin ELISA (ALPCO Diagnostics, Salem, NH). Secreted insulin was normalized to total protein content.

Statistical analysis

All values were expressed as means ± SEM with the number of independent individual experiments (biological replicates) presented in the figure legends. The different groups were compared by unpaired two-tail Students t-test. P value<0.05 was considered statistically significant.

Results

mTORC1 is hyper-activated in diabetic islets

In order to examine the pathological relevance of mTORC1 activation in type 2 diabetes, we investigated whether mTORC1 activity was altered in islets isolated from patients with type 2 diabetes. Activation of mTORC1 was detected by phosphorylation analysis of its downstream targets S6K1 at Thr 389 (pS6K), the direct S6K substrate ribosomal protein S6 at Ser 235/236 (pS6) and 4E-BP1 at Thr 37/46 (p4E-BP1) (Figure 1a). Representative Western blots of pS6 and p4E-BP1 from human islet isolations show that mTORC1 activity was elevated in the islets from patients with type 2 diabetes (Figure 1b). To thoroughly examine the activation of mTORC1 in control and diabetic islets, we analyzed pS6 and p4E-BP1 signals by quantitative densitometry of immunoblots from 10 non-diabetic and 8 diabetic individuals (see table 1). Our data show that the mTORC1 activity was significantly increased in type 2 diabetic islets compared to the controls (pS6: 1.76 fold; p4E-BP1: 3.07 fold, $p<0.05$; Figure 1c). To mimic a type 2 diabetes-like condition, isolated human islets were chronically cultured with elevated glucose concentrations for 72 hours. Diabetes-associated elevated glucose profoundly up-regulated mTORC1 activity in isolated human islets (Figure 1d,e) as

demonstrated by increased phosphorylation of S6K1, S6 and 4E-BP1. Together, these data show that a diabetic milieu robustly up-regulates mTORC1 activity in human islets.

Pancreatic beta cells are the source of up-regulated mTORC1 in human type 2 diabetic islets

As pancreatic islets are a composite of many cell types including insulin-expressing beta and glucagon-expressing alpha cells, we aimed to determine whether increased mTORC1 activity is present in beta or non-beta cells. Cell-type specific expression of activated mTORC1 in human islets was examined by immunostaining of pS6 (Ser 235/236). Immunofluorescent labelling of pS6 in combination with insulin or glucagon revealed higher mTORC1 activity in type 2 diabetic human islets with the presence of activated S6 predominantly in insulin-expressing beta cells (Figure 2a,c) and very low expression in glucagon-expressing alpha cells (Figure 2b,c). Quantification of insulin or glucagon co-expressing pS6 in isolated human islet sections of multiple non-diabetic controls and T2D individuals showed significant up-regulation of activated S6 in beta cells and thus confirm higher mTORC1 activity in type 2 diabetic islets indeed in the beta cell with insignificant expression in alpha cells (Figure 2c). mTORC1 activity was also analyzed in the established beta cell line INS-1E to provide comparison to primary human islets in terms of response to chronically elevated glucose concentrations. Prolonged culture of INS-1E cells with elevated glucose concentrations (22.2 mM) led to robust up-regulation of mTORC1 as represented by increased phosphorylation of S6K1, S6 and 4E-BP1 (Figure 2d,e) confirming that mTORC1 hyper-activation indeed occurs in the beta cell.

Reciprocal regulation of different mTOR complexes in diabetic islets

The activity and the functional status of the two different mTOR complexes was then examined by immunoprecipitation of endogenous mTOR and subsequent analysis of the mTOR-associated proteins raptor (representing mTORC1 activity) and rictor (representing mTORC2 activity). While endogenous raptor co-precipitated with mTOR (raptor-mTOR complex) was highly increased and confirmed higher activity of mTORC1 under high glucose conditions, rictor co-precipitated with mTOR (rictor-mTOR complex) was drastically reduced upon prolonged culture of human islets with high glucose suggesting a reciprocal regulation

of the two mTOR complexes in metabolically stressed human islets (Figure 3a-c). In order to confirm a functional decline of mTORC2 in diabetic islets, we investigated whether mTORC2 activity was changed in islets isolated from patients with type 2 diabetes. Activation of mTORC2 was assessed by phosphorylation analysis of its downstream targets AKT at Ser 473 (pAKT) and N-Myc Downstream Regulated 1 (NDRG1) at Thr 346 (pNDRG1) (Figure 4a). Consistent with the lower integrity of mTORC2 under high glucose, western blots analysis of pAKT and pNDRG1 from human islets show that mTORC2 activity was diminished in the islets from patients with type 2 diabetes (Figure 4b). To thoroughly examine the activation of mTORC2 in control and diabetic islets, we analysed pAKT and pNDRG1 signals by quantitative densitometry of immunoblots from 8 non-diabetic and 7 diabetic individuals. Our data show that the mTORC2 activity was significantly reduced in type 2 diabetic islets compared to nondiabetic controls (pAKT: 77% & pNDRG1: 53% reduction, $p<0.05$; Figure 4c).

mTORC1-S6K1 inhibition improved insulin secretion in type 2 diabetic islets

Such mTORC1 up-regulation correlates with beta cell dysfunction in human type 2 diabetic islets [14, 15]. Knockdown of the critical component of mTORC1, Raptor, is sufficient to increase insulin secretion in rodent beta cell lines [16]. We then hypothesized that mTORC1 inhibition improves beta cell function in already diabetic islets in the *ex vivo* setting. To date, no selective mTORC1 inhibitor has been discovered. Although rapamycin has widely been used to block mTORC1, it also inhibits mTORC2 [17-19]. Furthermore, the effects of rapamycin on islet biology are complex and controversial, with many laboratories reporting detrimental effects of rapamycin on beta cell function and survival [20] . Nevertheless, we had tested the effect of different concentrations of rapamycin in human type 2 diabetic islets. Rapamycin at both tested concentrations (10 and 20 nmol/l) had no effect on GSIS in T2D islets (data not shown). This as well as previous results makes rapamycin an inappropriate approach to target mTORC1 in human islets.

As S6K is one of the major downstream effectors of mTORC1, we have tested PF-4708671, a novel cell-permeable piperazinyl-pyrimidine compound, recently characterized as selective S6K1 inhibitor [21], as alternative approach to target mTORC1. The efficiency of PF-4708671

to block S6K signaling was confirmed in multiple isolated human islets preparations (Figure 5c). Human islets isolated from nondiabetic controls and patients with type 2 diabetes were treated with 10 µmol/l PF-4708671 for 4 hours. While short-term inhibitor treatment had no effect on GSIS in non-diabetic human islets (Figure 5a), it improved glucose-stimulated insulin response by 2.3-fold, compared to non-treated type 2 diabetic islets. Such functional restoration occurred independently in all tested human islet batches isolated from 3 patients with type 2 diabetes (Figure 5b), showing a beneficial effect of mTORC1-S6K1 signaling blockade in type 2 diabetic islets.

While mTORC2 is critical for maintaining beta cell function [22], chronic hyper-activation of mTORC1 diminishes mTORC2-AKT signaling through multiple negative feedback loops [23-25]. Thus, we checked whether mTORC1 inhibition can restore depleted mTORC2 in human T2D islets. In line with our insulin secretion data, S6K1 inhibitor increased mTORC2 activity as shown by increased phosphorylation of AKT demonstrated independently in human islets isolated from three donors with type 2 diabetes (representative Figures show results from 3 isolations; Figure 5c,d). This suggests that successful inhibition of the mTORC1-S6K1 axis improved mTORC2 function most probably by restraining mTORC1-mediated negative feedback loops.

In order to further corroborate the detrimental impact of hyper-activated mTORC1 on beta cell function in diabetic islets, we selectively inhibited mTORC1 by targeting its central component Raptor. Targeted inhibition of endogenous mTORC1 by siRNA-mediated silencing of Raptor resulted in substantially increased GSIS (Figure 5e,f) and efficiently reduced mTORC1 signaling (Figure 5g) confirming the inhibitory action of up-regulated mTORC1 in human type 2 diabetic islets.

S6K1 inhibition improved insulin secretion in isolated islets from obese diabetic mice

A progressive decline of beta cell function leading to beta cell exhaustion, loss of compensatory beta cell adaptation, glucose intolerance and type 2 diabetes in response to long-term high fat/ high sucrose diet have been clearly established in mice [10]. In order to further confirm the beneficial effect of mTORC1-S6K1 blockade on beta cell function, we have tested whether acute S6K1 inhibition can improve insulin secretion of islets isolated

from HFD-treated mice for 17 weeks. Consistent with the improved beta cell function of human diabetic islets upon S6K1 inhibition, isolated HFD islets treated with S6K1 inhibitor showed significantly higher GSIS response compared to un-treated HFD islets (Figure 6a); the stimulatory index was highly improved (Figure 6b). Intriguingly and in line with human islets data, S6K1 inhibitor treatment highly increased mTORC2 activity as demonstrated by phosphorylation of AKT and NDRG1 (Figure 6c,d). Efficiency of S6K1 inhibition to restore mTORC2 activity has also been tested in isolated islets from leptin-receptor deficient db/db mice as another mouse model of type 2 diabetes. Inverse regulation of different mTOR complexes with higher mTORC1 and lower mTORC2 activities is reproduced in pancreatic islets of diabetic db/db mice (Figure 6e) which confirms previously published similar observations in the db/db islets [8] [26]. S6K1 inhibitor treatment fully blocked S6 phosphorylation and normalized mTORC2 activity in isolated db/db islets (Figure 6e) confirming mTORC1-S6K1-mediated mTORC2 depletion as possible pathogenic signaling hallmark of diabetic islets.

Discussion

The beta cells' loss in its response to chronically elevated nutrients and resultant deficit in beta cell function and mass represents one of the most fundamental pathological hallmarks of type 2 diabetes. Despite the finding of constitutive mTORC1 activity in the pancreatic islets of mouse models of type 2 diabetes [8], hyperactivity of mTORC1 was unknown in human type 2 diabetic islets so far. This study provides the first direct evidence that type 2 diabetic islets as well as metabolically stressed human islets display an opposite regulation of mTORC1/2 signals with higher mTORC1 and lower mTORC2 activities. Inhibition of mTORC1 signaling either through S6K1 inhibition or knockdown of Raptor improved insulin secretion of human and mouse type 2 diabetic islets. This observation provides a mechanism for the collapse of the beta cell's ability for functional and mass adaptation to excess nutrients during obesity. Subsequently, type 2 diabetes develops with hyperglycemia and relative insulin deficiency.

Pancreatic beta cell-specific TSC2 knockout mice, in which mTORC1 is constitutively active, show a biphasic pattern of beta cell turnover [8, 27]. While sustained mTORC1 activation

leads to hyperinsulinemia and improved glucose homeostasis by enhancing beta cell hypertrophy and hyperplasia in young mice, prolonged constitutive mTORC1 hyper-activation diminishes pancreatic beta cell mass by inducing apoptosis leading to progressive hyperglycemia and the development of diabetes in older mice. This cell-autonomous biphasic regulatory pattern of mTORC1 function can explain, at least in part, signaling alterations in the widely accepted model of beta cell deterioration “compensation/ decompensation switch” during the progression of type 2 diabetes. In the first functional compensatory phase, metabolic drivers such as insulin resistance and nutrient excess increase beta cell mTORC1 activity; this correlates with higher insulin production as well as compensatory beta cell hypertrophy and hyperplasia proposing mTORC1 as a key positive regulator of beta cell function and mass [28]. However, in the second detrimental de-compensatory phase, chronic activation of mTORC1 caused by sustained nutrient over-load mainly by high glucose and NEFAs exposure leads to beta cell exhaustion, functional collapse and ultimate cell death. The higher mTORC1 activity detected in the islets of individuals with type 2 diabetes in our study is consistent with observations from animal models of type 2 diabetes and maybe the consequence of nutrient overload in beta cells as one main characteristic feature of pancreatic islets in type 2 diabetes.

How does chronic activation of mTORC1 promote impaired beta cell function and survival? It is firmly established that mTORC1 mediates potent negative feedback loops that restrain upstream mitogenic signaling from insulin/IGF receptor pathways [23-25]. These loops cause long-term compensatory mechanisms inhibiting AKT kinase via multiple mechanisms, e.g. mTORC1/S6K suppresses AKT signaling through phosphorylation of IRS1/2, Grb10 and Sin1 proteins [23-25]. Notably, chronic mTOR activation negatively regulates beta cell survival by direct IRS2 phosphorylation and subsequent proteasomal degradation [25]. Consistently, rapamycin-mediated mTORC1 inhibition up-regulates IRS2 and AKT phosphorylation in mouse islets *in vivo* and *in vitro* suggesting the presence of the negative functional loop from mTORC1 that prevents IRS2-AKT signaling [29]. A novel regulatory negative feedback loop was established through mTORC1/S6K-mediated phosphorylation of the mTORC2 subunit Sin1 [24], inhibiting the mTORC2-AKT signaling axis which is essential for maintaining normal beta cell mass [22]. This may explain the defective Akt-Ser473

phosphorylation (site of mTORC2 phosphorylation) seen in stressed beta cells [10, 26] and type 2 diabetic islets in our study and may be associated with the impaired mTORC2 complex integrity under prolonged glucose stimulation as presented here. AKT is the master pro-survival kinase in the beta cells [10, 22, 28, 30]. One major detrimental outcome of the negative feedback loops initiated by constitutive mTORC1 hyper-activity would be impaired AKT-mediated pro-proliferative and pro-survival responses. Indeed, defective AKT signaling is a hallmark of diabetic beta cells in the context of type 2 diabetes [10, 26]. Using various *ex vivo* experimental models of diabetes including isolated human islets from patients with type 2 diabetes as well as from hyperglycaemic HFD and db/db mice, we demonstrated that inhibition of mTORC1-S6K1 signaling leads to striking restoration of mTORC2 activity; this suggests the existence of functional inhibitory loop(s) from mTORC1 to mTORC2. We speculate that such negative feedback mechanisms triggered by mTORC1-S6K1 hyper-activation may operate *in vivo* in type 2 diabetic beta cells. The identification of specific pathway(s) down-stream of activated mTORC1, which are involved in the decline of mTORC2 signaling, may provide a better understanding of such complex interplay and some clues to its reversal.

Another so far unexplored but important mechanistic link of mTORC1 activity and beta cell failure in diabetes is the impaired autophagy. Defective autophagy has been implicated in the process of beta cell failure in type 2 diabetes [27, 31, 32] and it is well established that mTORC1 hyper-activity results in inhibition of autophagy [27]. Mice deficient for autophagy-promoting protein 7 (Atg7), an essential component of autophagy, show exacerbated diabetes development under high fat diet by diminishing pancreatic beta cell mass and function [31]. Intriguingly, type 2 diabetic islets accumulate autophagic vacuoles and autophagosomes concomitant with an increase in apoptotic beta cell death [33] suggesting an impaired flux of autophagy. The aberrant activation of mTORC1 in type 2 diabetic islets may provide a unique explanation for the chronic deterioration of the physiologically protective autophagy pathway during the course of beta cell failure.

Despite the prevailing view of mTORC1 inhibition for type 2 diabetes therapy, studies using mTOR inhibitors such as rapamycin show controversial results. While rapamycin suppresses stress-induced apoptosis in beta cell lines [9, 34], improves beta cell function and thus

corrects glucose homeostasis in Akita mouse model of type 2 diabetes [35], chronic inhibition of mTORC1 by rapamycin causes glucose intolerance in mice [19, 29]. This is due to the fact that chronic inhibition of mTORC1 with rapamycin disrupts mTORC2 which is crucial for the insulin-mediated suppression of hepatic gluconeogenesis [19] and for maintaining pancreatic functional beta cell mass [22]. Alternatively, our data show that blocking of mTORC1-S6K1 signaling by the highly specific S6K1 inhibitor enhanced insulin secretion in human and mouse type 2 diabetic islets *ex vivo* suggesting that elevated mTORC1 activity seen in our study negatively regulates beta cell function. Interestingly, *Sham et al* reported [36] that rapamycin treatment compromised glucose metabolism and failed to improve AKT phosphorylation in liver, fat and muscle of HFD-treated mice, but in contrast, S6K1 inhibition by PF-4708671 improved glucose tolerance and corrected HFD-induced impaired AKT phosphorylation in metabolically active tissues of obese mice. All these previous data together with our study in human islets suggest the mTORC1/S6K1 axes as potential therapeutic target for treatment of type 2 diabetes.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Author contribution

Conceived the project, AA; designed and performed experiments and analyzed data, TY,

SR, KG, BL, AA; contributed reagents or analytic tools: JO; wrote the paper and supervised the project, AA, KM. All authors critically reviewed the manuscript for important intellectual content and approved the final version to be published. AA and KM are the guarantors of this work.

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592-603

Legends

Figure 1. mTORC1 is hyper-activated in diabetic islets. (a) Scheme depicting downstream targets of mTORC1. Activation of mTORC1 leads to phosphorylation and inactivation of 4E-BP1 (pT37/46) and activation of S6K (pT389). Active S6K phosphorylates its downstream target ribosomal protein S6 (pS235/236). (b,c) Human islets isolated from 10 non-diabetic controls (*n*=10) and 8 individuals with type 2 diabetes (*n*=8) were analyzed for mTORC1 activity; (b) representative western blot and (c) quantitative densitometry analysis of pS6 and p4E-BP1 signals (white bars, control islets; grey bars, type 2 diabetic (T2D) islets). (d,e) Human islets were treated with 22.2 mmol/l glucose for 3 days. (d) Western blots and (e) quantitative densitometry analysis of pS6K, pS6 and p4E-BP1 signals from three non-diabetic controls (n=3) are shown (white bars, control human islets; grey bars, high glucose-treated human islets). Data are pooled from three independent experiments (*n*=3). pS6, pS6K and p4E-BP1 were analyzed by western blotting. Actin/GAPDH was used as loading control. Data show means ± SE. *p<0.05 compared to non-diabetic controls (c), or 5.5 mmol/l glucose treated control human islets (e).

Figure 2. mTORC1 is hyper-activated in diabetic beta cells. (a,b) Freshly isolated human islets of non-diabetic individuals as well as from patients with T2D were cultured overnight. Fixed paraffin-embedded islet sections were double-stained for pS6 (red) and (a) insulin (green) or (b) glucagon (green). Representative images from two non-diabetic control islets and two type 2 diabetic islets shown. (c) Quantitative analysis of insulin- or glucagon-co-expressing pS6 in human islet sections from controls and T2D individuals. Data are means ± SEM from 3 non-diabetic controls (*n*=3) and 3 individuals with type 2 diabetes (*n*=3). 2700 and 1725 insulin-positive beta cells and 1493 and 1585 glucagon-positive alpha cells were counted for controls and T2D donors, respectively. Black bars, pS6-insulin co-positive cells; grey bars, pS6-glucagon co-positive cells. *p<0.05 compared to non-diabetic controls. (d,e) INS-1E cells were treated with 22.2 mmol/l glucose for 2 days. (d) Western blot and (e) quantitative densitometry analysis of pS6K, pS6 and p4E-BP1 signals are shown as means ± SEM (white bars, control INS-1E cells; grey bars, high

glucose-treated INS-1E cells). Actin was used as loading control. *p<0.05 compared to 11.1 mmol/l glucose treated control INS-1E cells.

Figure 3. Reciprocal regulation of mTORC1/2 complex integrity by chronically elevated glucose in human islets. (a) Human islets were treated with the 22.2 mmol/l glucose for 3 days. Co-immunoprecipitations were performed using anti-IgG and anti-mTOR antibodies and western blots of precipitates and input fractions were analyzed for Raptor, Rictor and mTOR. "Input" is the total islet protein lysate before immunoprecipitation. (b,c) Pooled quantitative densitometry analysis of co-precipitated Raptor (b) or Rictor (c) with mTOR from 3 independent experiments from 3 different human islets donors (*n*=3). Data show means ± SE. *p<0.05 compared to 5.5 mmol/l glucose treated control human islets.

Figure 4. mTORC2 activity is diminished in diabetic islets. (a) Scheme depicting downstream targets of mTORC2. Activation of mTORC2 leads to phosphorylation and activation of AKT (pS473) and SGK1 (pS422). Active SGK1 phosphorylates its downstream target NDRG1 (pT346). (b) Representative western blot and (c) quantitative densitometry analysis of pNDRG1 and pAKT signals. Human islets isolated from eight non-diabetic controls (*n*=8) and seven individuals with T2D (*n*=7) were analysed for mTORC1 activity. pNDRG1 and pAKT were analysed by western blotting. GAPDH was used as loading control (white bars, control islets; grey bars, T2D islets). Data show means ± SE. *p<0.05 compared to non-diabetic controls (c).

Figure 5. S6K1 inhibitor improved insulin secretion in islets from patients with type 2 diabetes. (a) Isolated human islets from non-diabetic individuals (3 donors; 12-16 technical replica per group) and (b) patients with T2D (3 donors; 8-10 technical replica per group) were left untreated or treated with 10 µmol/l PF-4708671 (S6K1 inhibitor) for 4 hours. (a,b) Insulin secretion during 1h-incubation with 2.8 mmol/l (basal) and 16.7 mmol/l glucose (stimulated), normalized to protein content (white bars, basal insulin; grey bars, stimulated insulin). (c,d) Human islets isolated from 3 individuals with T2D (*n*=3) were analyzed for pS6 and pAKT. (c) Western blots of 3 different human islets donors and (c) quantitative densitometry analysis of

pS6 and pAKT signals (white bars, untreated human islets; grey bars, S6K1 inhibitor-treated human islets). (e-g) Isolated human islets from patients with T2D (2 donors; 6 technical replica per group) were transfected with control siScr or raptor siRNA (siRaptor). (e) Insulin secretion during 1h-incubation with 2.8 mmol/l (basal) and 16.7 mmol/l glucose (stimulated), normalized to protein content (white bars, basal insulin; grey bars, stimulated insulin). (f) The insulin stimulatory index denotes the ratio of secreted insulin during 1h-incubation with 16.7 mmol/l and 2.8 mmol/l glucose respectively (white bars, basal insulin; grey bars, stimulated insulin). (g) Representative western blots of Raptor, pS6K, pS6 and p4E-BP1 is shown. Both lanes were run on the same gel but were noncontiguous. Data show means ± SE.

Figure 6. S6K1 inhibitor improved insulin secretion in islets from diabetic mice. (a-d) (a,b) Insulin secretion during 1-h incubation with 2.8 mmol/l (basal) and 16.7 mmol/l (stimulated) glucose normalized to protein content from isolated islets from HFD-treated C57BL/6J mice for 17 weeks, cultured overnight, treated with or without 10 µmol/l PF-4708671 (S6K1 inhibitor) for 4 hours and subjected to an *in vitro* GSIS assay (*n*=8 per group). The insulin stimulatory index denotes the ratio of secreted insulin during 1-h incubation with 16.7 mmol/l to secreted insulin at 2.8 mmol/l glucose. (c,d) Isolated islets were analyzed for pS6, pAKT and pNDRG1. (c) Western blot of 3 different isolations and (d) quantitative densitometry analysis of pS6, pAKT and pNDRG1 signals (white bars, untreated HFD islets; grey bars, S6K1 inhibitor-treated HFD islets). (e) Islets isolated from diabetic 12-week old db/db mice and their heterozygous non-diabetic littermate controls (db/+) were left untreated or treated with S6K1 inhibitor for 4 hours. (g) pS6, pAKT and pNDRG1 was analyzed by Western blot. Data show means ± SE. *p<0.05 compared to HFD islets.

Donors	AGE	BMI	Gender	HbA1c %	HbA1c mmol/mol	Cause of death
T2D	37	39.3	F	7.3	57	anoxia
	48	41	M	6.5	48	unknown
	56	24.3	M	5.1	32	stroke
	54	29	M	6.5	48	vascular/hypertension
	57	36.3	F	8.2	66	cerebrovascular/stroke
	48	30.2	M	7.9	63	trauma
	56	40	F	8.1	65	cerebrovascular/stroke
	59	29.2	F	7.4	57	anoxia, CPR
	51	24.4	M	6.9	52	stroke
	58	39.3	M	8.9	74	anoxia
	61	28	M	5.2	33	cerebrovascular
Mean	**53.2**	**32.9**				
SE	1.9	1.8				
Controls	23	24.5	F	4.9	30	head trauma
	51	24.4	F	<6	<42	head trauma
	31	23.8	M	<6	<42	suicide (gunshot)
	48	25	M	<6	<42	stroke
	54	37.3	F	4.9	30	meningitis
	51	27.5	M	<6	<42	unknown
	26	46.6	M	4.8	29	head trauma
	56	33.1	F	<6	<42	cerebrovascular/stroke
	82	22.2	F	<6	<42	vascular event
	64	27.8	M	5.2	33	stroke
	57	23.1	F	<6	<42	stroke
	38	34	F	5	31	anoxia
	51	24.1	M	<6	<42	stroke
Mean	**47.8**	**28.0**				
SE	4.3	1.8				

Table 1. Human islet donor characteristics.
F, female; M, male. CPR, cardiopulmonary resuscitation

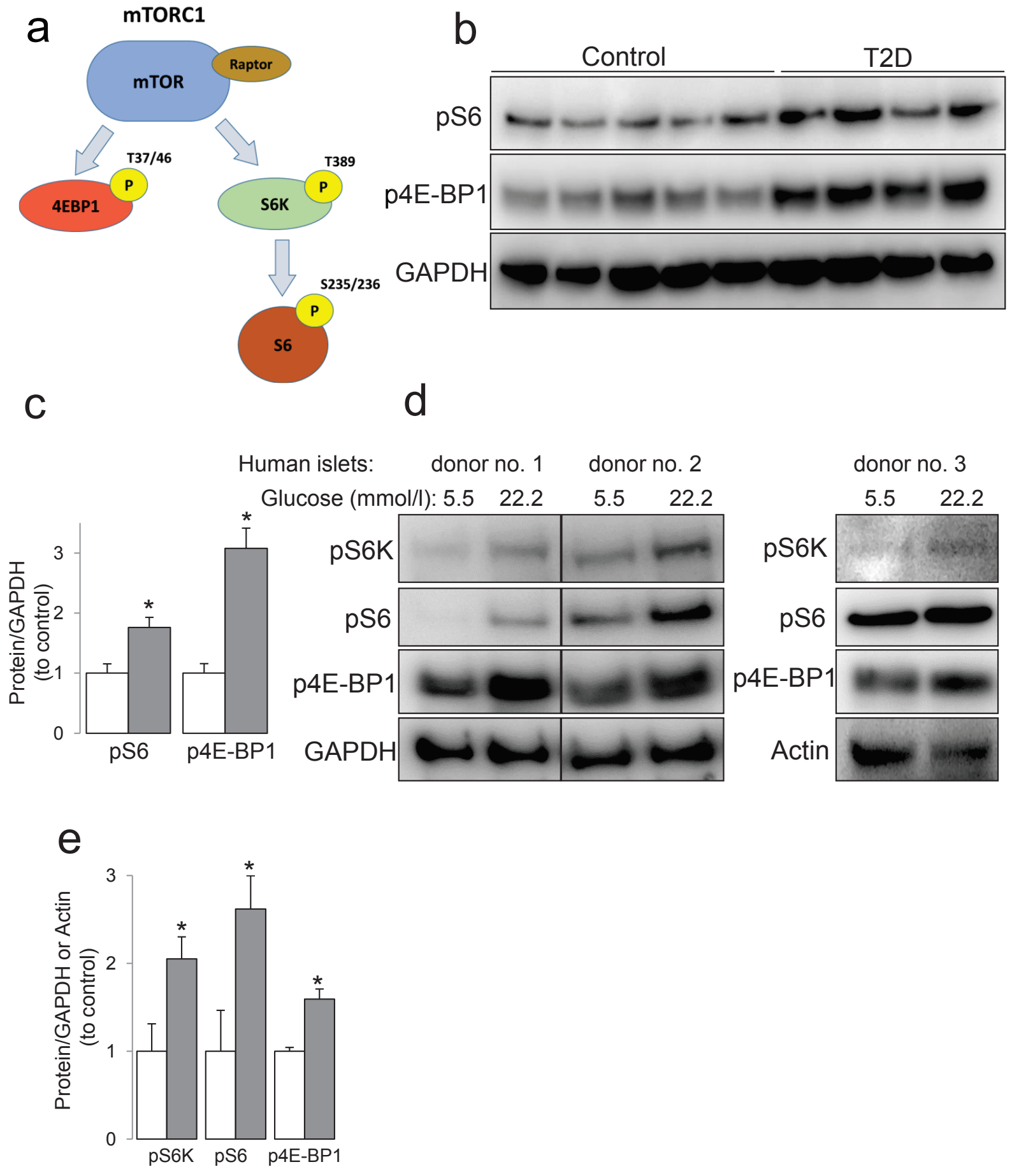

a
mTORC1
mTOR
Raptor
T37/46
P
4EBP1
T389
P
S6K
S235/236
P
S6
b
Control
T2D
pS6
p4E-BP1
GAPDH
c
Protein/GAPDH (to control)
0
1
2
3
*
*
pS6
p4E-BP1
d
Human islets:
donor no. 1
donor no. 2
donor no. 3
Glucose (mmol/l): 5.5
22.2
5.5
22.2
5.5
22.2
pS6K
pS6
p4E-BP1
GAPDH
pS6K
pS6
p4E-BP1
Actin
e
Protein/GAPDH or Actin (to control)
0
1
2
3
*
*
*
pS6K
pS6
p4E-BP1

Figure 1

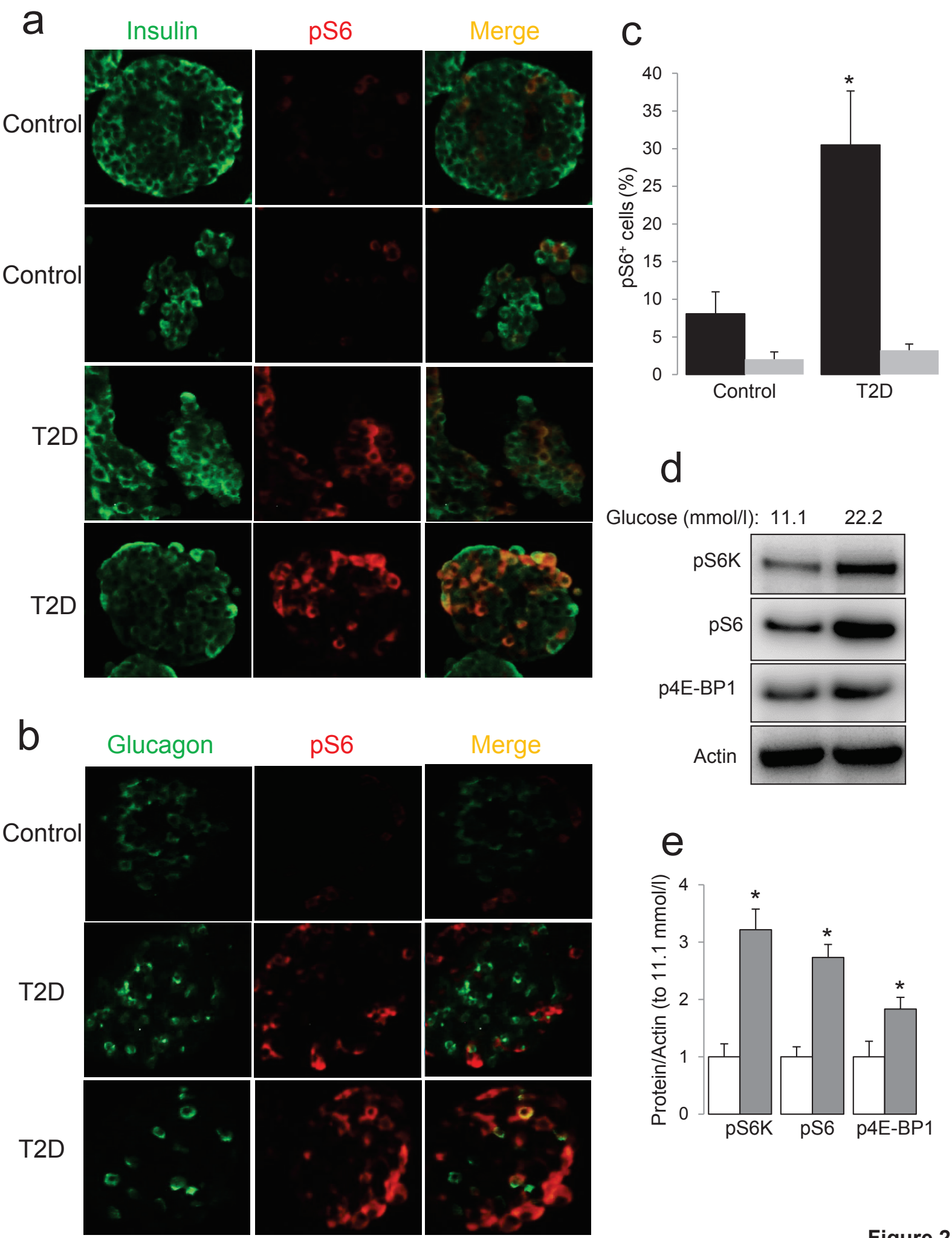

a
Insulin
pS6
Merge
Control
Control
T2D
T2D
b
Glucagon
pS6
Merge
Control
T2D
T2D
c
pS6+ cells (%)
40
35
30
25
20
15
10
5
0
*
Control
T2D
d
Glucose (mmol/l): 11.1 22.2
pS6K
pS6
p4E-BP1
Actin
e
Protein/Actin (to 11.1 mmol/l)
4
3
2
1
0
*
*
*
pS6K
pS6
p4E-BP1

Figure 2

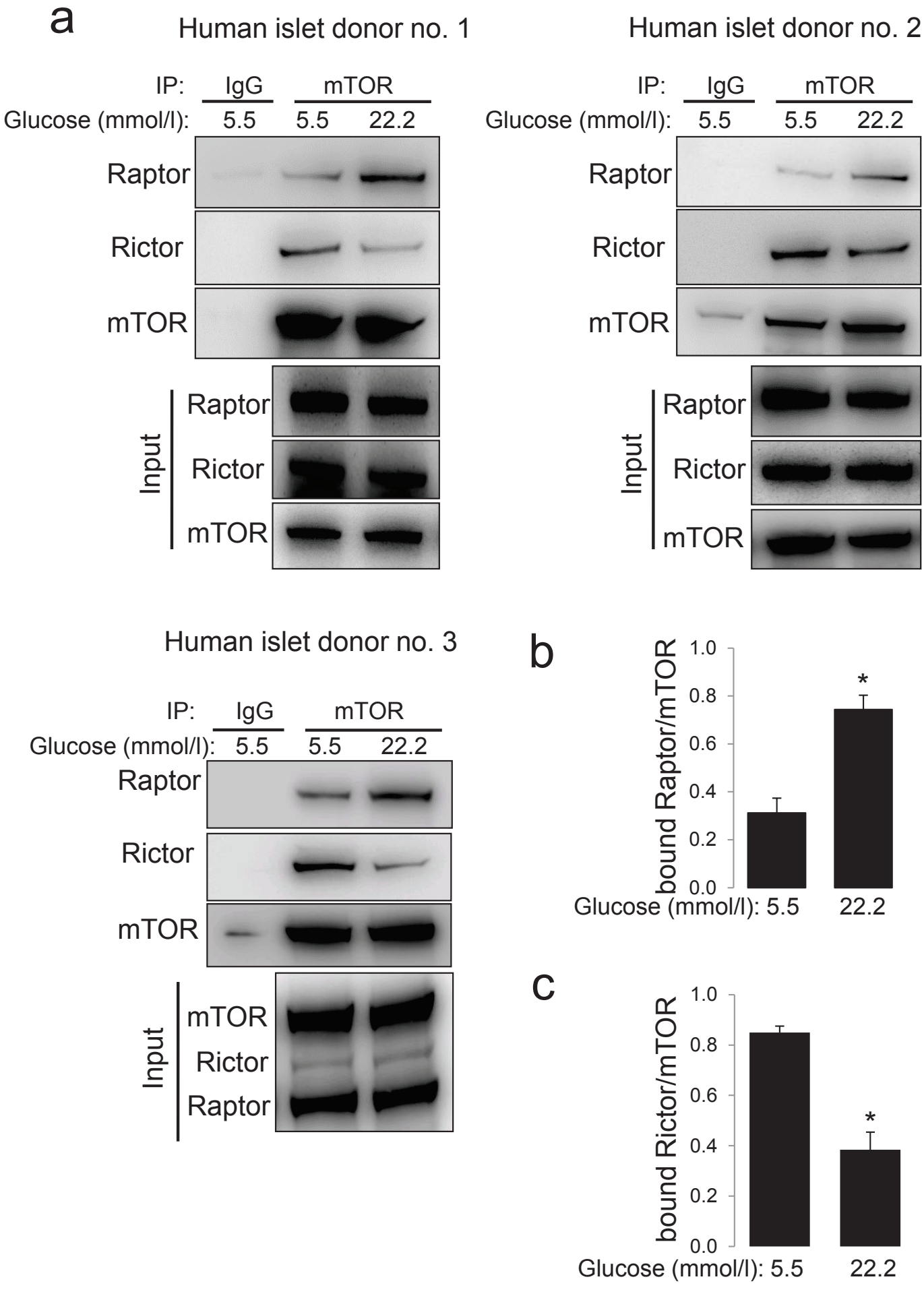
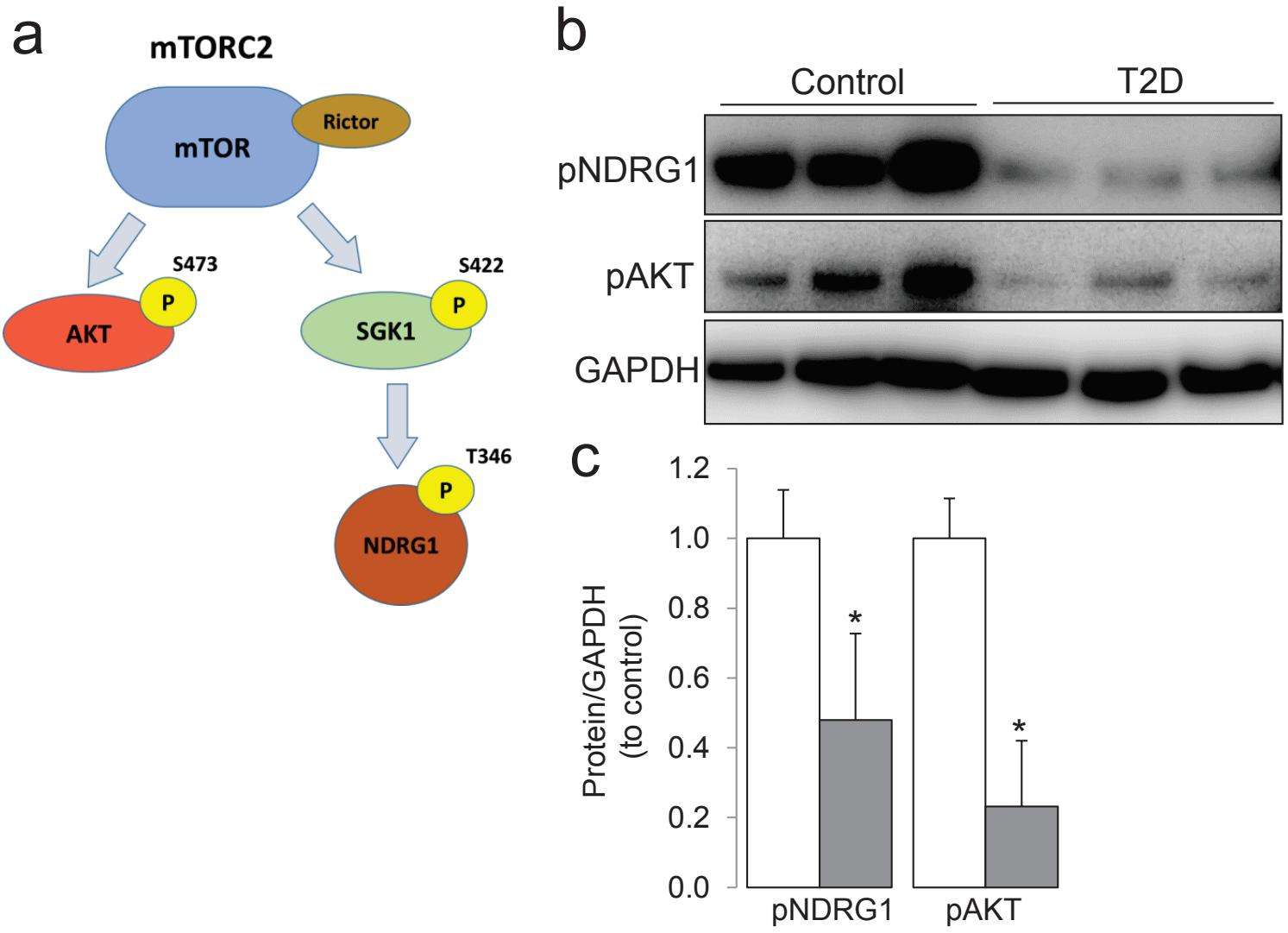

a
Human islet donor no. 1
IP: IgG mTOR
Glucose (mmol/l): 5.5 5.5 22.2
Raptor
Rictor
mTOR
Input
Raptor
Rictor
mTOR
Human islet donor no. 2
IP: IgG mTOR
Glucose (mmol/l): 5.5 5.5 22.2
Raptor
Rictor
mTOR
Input
Raptor
Rictor
mTOR
Human islet donor no. 3
IP: IgG mTOR
Glucose (mmol/l): 5.5 5.5 22.2
Raptor
Rictor
mTOR
Input
mTOR
Rictor
Raptor
b
bound Raptor/mTOR
1.0
0.8
0.6
0.4
0.2
0.0
*
Glucose (mmol/l): 5.5 22.2
c
bound Rictor/mTOR
1.0
0.8
0.6
0.4
0.2
0.0
*
Glucose (mmol/l): 5.5 22.2

Figure 3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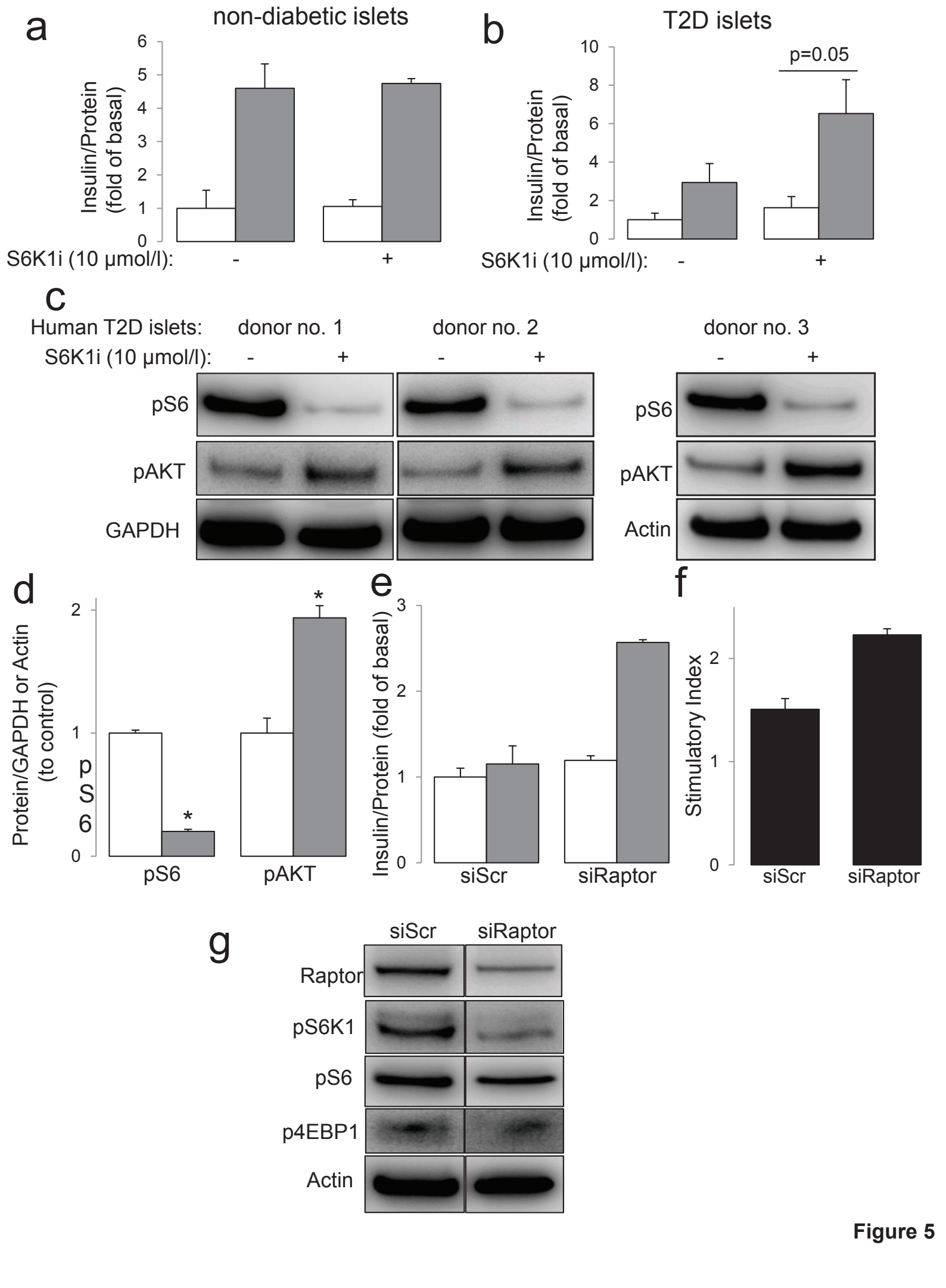
mTORC2
mTOR
Rictor
S473
P
AKT
S422
P
SGK1
T346
P
NDRG1
b
Control
T2D
pNDRG1
pAKT
GAPDH
c
Protein/GAPDH
(to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
pNDRG1
pAKT

Figure 4


a
non-diabetic islets
Insulin/Protein (fold of basal)
S6K1i (10 µmol/l): - +
b
T2D islets
p=0.05
Insulin/Protein (fold of basal)
S6K1i (10 µmol/l): - +
c
Human T2D islets: donor no. 1 donor no. 2 donor no. 3
S6K1i (10 µmol/l): - + - + - +
pS6
pAKT
GAPDH
pS6
pAKT
Actin
d
Protein/GAPDH or Actin (to control)
pS6
pS6
pAKT
e
Insulin/Protein (fold of basal)
siScr
siRaptor
f
Stimulatory Index
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siRaptor
g
siScr
siRaptor
Raptor
pS6K1
pS6
p4EBP1
Actin

Figure 5

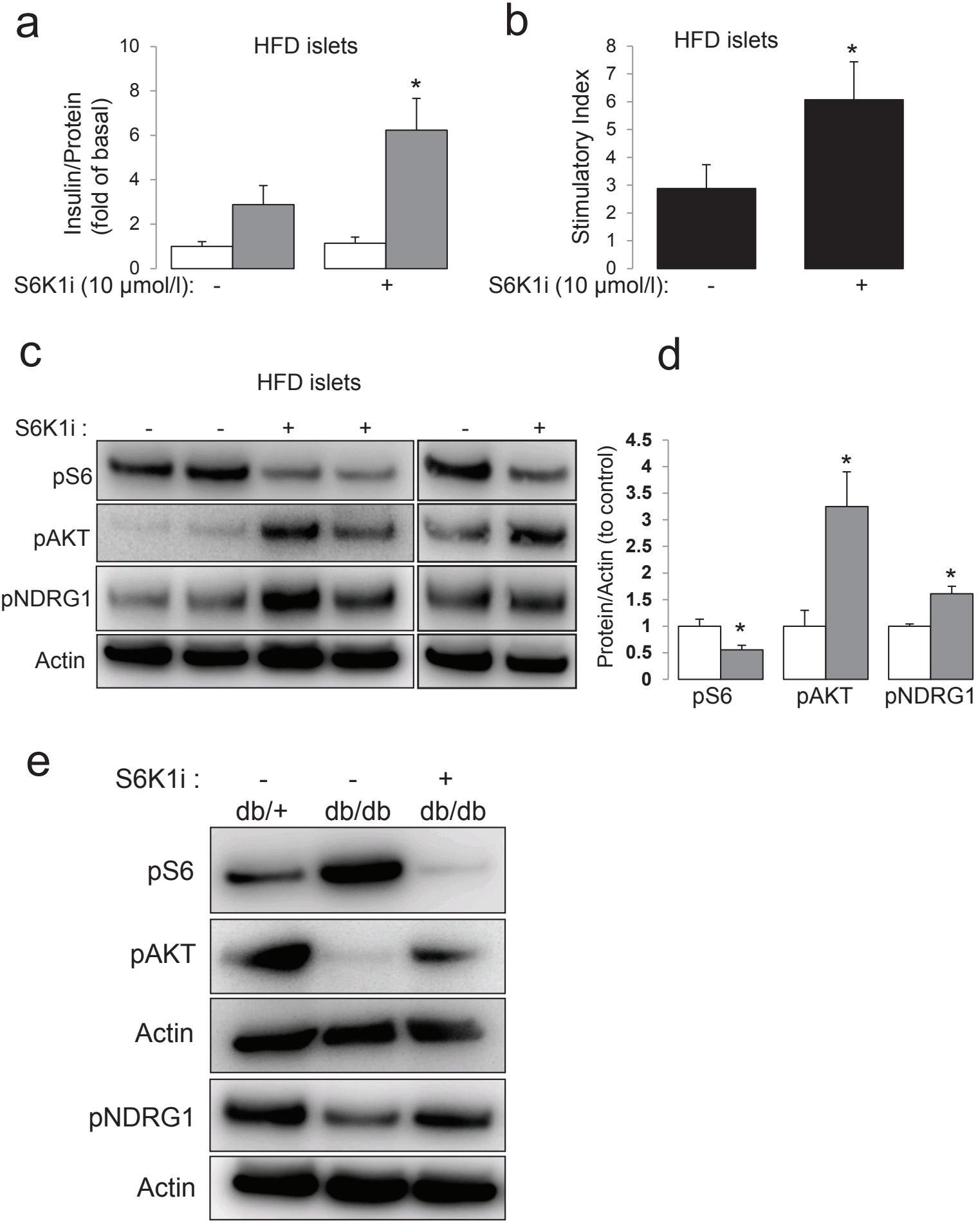
a
HFD islets
Insulin/Protein (fold of basal)
10
8
6
4
2
0
*
S6K1i (10 µmol/l): -
+
b
HFD islets
Stimulatory Index
8
7
6
5
4
3
2
1
0
*
S6K1i (10 µmol/l): -
+
c
HFD islets
S6K1i : - - + + - +
pS6
pAKT
pNDRG1
Actin
d
Protein/Actin (to control)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
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Figure 6

Manuscript IV

2.4 The Hippo kinase LATS2 impairs pancreatic β-cell survival and function in diabetes

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Contribution:

Designed and performed all experiments, analyzed data and wrote the paper.

The Hippo kinase LATS2 impairs pancreatic β-cell survival and function in diabetes

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Abstract

Both type 1 and type 2 diabetes mellitus result from an absolute or relative decline in pancreatic β-cell function and/or mass. Apoptosis and loss of function are hallmarks of β-cell failure and the fundamental cause of diabetes, but the molecular mechanisms underlying theses pathological defects are poorly understood. Here we report that overexpression of Large-tumor suppressors 2 (LATS2), a core component of the Hippo signaling pathway, is sufficient to induce β-cell apoptosis and impaired function. Conversely, LATS2 deficiency in β-cells and primary isolated human islets as well as LATS2 ablation in mice reduced β-cell apoptosis and ameliorated diabetes development. We showed that LATS2 activated mechanistic target of rapamycin complex 1 (mTORC1) by suppression of AMP-activated protein kinase (AMPK) signaling. We also observed that genetic and pharmacological modulation of mTORC1 and AMPK signaling pathways controlled the pro-apoptotic outcome of activated LATS2 and thereby constituted a stress-sensitive survival pathway regulating the β-cell apoptosis program. Consequently, β-cell apoptosis triggered by chemically-induced defective autophagy was exacerbated and suppressed by LATS2 overexpression and knockdown, respectively. Our data reveal an important role for LATS2 in pancreatic β-cell apoptosis and suggest LATS2 as potential therapeutic target to improve pancreatic β-cell survival in diabetes.

Key words：Apoptosis, Large tumor suppressor 2 (LATS2); Hippo signaling pathway; Mammalian target of rapamycin complex 1 (mTORC1); AMP-activated protein kinase (AMPK); Autophagy; Diabetes

Introduction

Diabetes is a major health problem worldwide. Both type 1 diabetes (T1D) and type 2 diabetes (T2D) result from an absolute or relative decline in pancreatic β-cell function and/or mass [1]. Apoptosis of insulin-producing β-cells is the hallmark of both T1D and T2D [2-5]. In both cases the mechanisms of β-cell apoptosis are complex and as yet poorly understood. T1D is an autoimmune disease resulting from selective destruction of pancreatic islet β-cells [6]. T2D is a complex metabolic disorder characterized by insulin resistance as well as decreased insulin secretory function and ultimately reduced β-cell mass, resulting in the development of chronic β-cell dysfunction and relative insulin deficiency [7, 8]. β-cell failure is caused by multiple stimuli including glucotoxicity, lipotoxicity [9, 10], amyloid deposition [11] and inflammation [12], which can trigger endoplasmic reticulum stress and/or oxidative stress [13, 14]. Thus, multiple triggering factors initiate a variety of signaling cascades that affect the expression of apoptotic genes and subsequent β-cell failure. Given the varied and enigmatic nature of the causes of β-cell failure, inhibition of apoptosis and/or β-cell dysfunction represents a potential therapeutic intervention to the treatment of diabetes [1].

The identification of signaling mechanisms and molecular events that are responsible for cellular and molecular alterations during β-cell demise is instrumental for better understanding of the molecular changes underlying functional β-cell mass loss and development of therapeutic strategies for a β-cell-directed therapy in diabetes. The Hippo pathway -first discovered using genetic screens in *Drosophila*-is conserved in mammals and critical for regulation of organ size. Mammalian Sterile 20-like kinases (MST1/2) and Large-tumor suppressors (LATS1/2) represent core components of the mammalian Hippo pathway. MST1/2, in complex with a regulatory protein Salvador (Sav1), phosphorylates and activates LATS1/2 kinases, which also form a complex with a regulatory protein Mps-one binder 1 (MOB1). Transcriptional coactivator Yes-associated protein (YAP) is a terminal downstream effector of the Hippo pathway. YAP function mainly regulated by phosphorylation-dependent mechanism. Kinase

LATS1/2 inactivates YAP by direct phosphorylation at S127, enhancing YAP binding to 14-3-3 proteins, its cytoplasmic sequestration and subsequent proteasomal degradation [15-18]. In association with TEA domain (TEAD) family transcription factors, YAP fosters the expression of target genes, with pro-proliferative and anti-apoptotic outcomes [18-20].

As an emerging significant player in many diseases, the Hippo pathway has attracted increasing attention for the development of new drugs. While the role of Hippo signaling in tumor development is becoming more and more important [21-25], non-cancer abnormalities involving Hippo components have only been studied to a limited extent [26-28]. We have recently identified MST1, the key component of Hippo signaling, as a novel regulator of pancreatic β-cell death and dysfunction in human and rodent β-cells *in vitro* as well as in diabetic animal models *in vivo* [28]. LATS2, a MST1 down-stream substrate, is a ubiquitously expressed serine/threonine kinase and involved in multiple cellular processes such as morphogenesis, proliferation, stress responses, apoptosis and differentiation [29-33]. LATS2 promotes cell death through regulation of multiple downstream targets such as P53, FOXO1, c-Abl and YAP [31, 34-37]. In this regard, the MST1-LATS2 axis is an important regulator of apoptosis in the heart: knockdown or genetic deletion of MST1 or LATS2 in cardiomyocytes provides protection against ischemic injury [33, 36, 38]. We have recently shown that loss of NF2/Merlin, an upstream regulator of Hippo pathway, protects pancreatic β-cells by inhibiting LATS2 activity [39]. In this study, we investigated the physiological role of LATS2 in the β-cell, whether its hyper-activation would trigger β-cell death and impaired insulin secretion and whether its deficiency would promote β-cell survival under diabetic conditions *in vitro* and *in vivo*.

Methods

Cell culture, treatment and islet isolation

Human islets were isolated from pancreases of healthy organ donors at the University of Illinois at Chicago, Lille University or ProdoLabs and cultured on extra cellular

matrix (ECM)-coated dishes (Novamed, Jerusalem, Israel) as described previously [40]. The clonal rat beta cell line INS-1E was provided by Dr. Claes. Wollheim (Geneva&Lund University). Human islets were cultured in complete CMRL-1066 (Invitrogen) medium at 5.5 mM glucose and INS-1E cells were cultured in complete RPMI-1640 medium at 11.1 mM glucose as described previously [28]. Human islets and INS-1E cells were exposed to complex diabetogenic conditions: 22.2 mM glucose, the mixture of 22.2 mM glucose plus 0.5 mM palmitic acid, the mixture of 2 ng/mL recombinant human IL-1β (R&D Systems, Minneapolis, MN) plus 1,000 U/ml recombinant human IFN-γ (PeProTech) for 48-72 h. In some experiments, cells and islets were additionally cultured with 100 nM Rapamycin or 10 μM S6K1 selective inhibitor PF-4708671 (Calbiochem) for 24h, 50 μM chloroquine (Sigma) or 20 nM Bafilomycin (Sigma) for 4h. Palmitic acid was dissolved as described previously [41]. Ethical approval for the use of human islets had been granted by the Ethics Committee of the University of Bremen.

Mice

β-cell-specific LATS2 knockout(β-LATS2$^{-/-}$) mice were generated by crossing mice harboring exon 4 of the LATS2 gene flanked by *loxP* sites (LATS2$^{fl/fl}$, provided by Dr. Dae-Sik Lim, Korea Advanced Institute of Science and Technology, South Korea [42]) with mice expressing Cre under the rat insulin-2 promoter (B6;D2-Tg(Ins-cre)23Herr:RIP-Cre [43], kindly provided by Dr. Susanne Ullrich (Medizinische Klinik, Universitätsklinikum, Tübingen). RIP-Cre-LATS2$^{fl/+}$mice were intercrossed to generate RIP-Cre-LATS2$^{fl/fl}$ (β-LATS2$^{-/-}$). For multiple low dose streptozotocin (MLD-STZ) experiments, 8- to 10-week old β-LATS2$^{-/-}$, flox control (LATS2$^{fl/fl}$) and flox-negative littermates (RIP-Cre) were injected with STZ for 5 consecutive days (40 mg/kg STZ or citrate buffer vehicle control). Random blood was obtained from the tail vein of non-fasted mice every 2 or 3 days, and glucose was measured using Glucometer (Freestyle; TheraSense, Alameda, CA). Mice were killed and pancreases were isolated at the end of experiment. All mice used in this experiment were male and housed in a temperature-controlled room with a 12-h

light-dark cycle and were allowed free access to food and water in agreement with US National Institutes of Health animal care guidelines and the German animal protection law and approved by the Bremen Senate.

Glucose tolerance test (GTT) and measurement of insulin release

For GTT, mice were fasted 12h overnight and injected with glucose (40%; B. Braun, Melsungen, Germany) at a dose of 1g/kg body weight. Blood samples were obtained at time points 0, 15, 30, 60, 90 and 120 min for glucose measurements using a glucometer. Insulin secretion was measured before (0 min) and after (15 min) i.p. injection of glucose (2g/kg body weight) and measured using ultrasensitive mouse Elisa kit (ALPCO Diagnostics, Salem, NH).

Plasmids and siRNAs

Myc-LATS2 and kinase dead Myc-LATS2 (LATS2-KD) were provided by Dr. Jixin Dong (Nebraska Medical Center, Omaha, NE) [44]. pLU-FLAG-Sestrin2 was provided by Dr.Andrei V.Budanov (Virginia Commonwealth University, Richmond, USA) [45]. pBABEpuro GFP-LC3 (22405) and pEBG-AMPKα1 (1-312) (27632) were obtained from Addgene. GFP was used as a control. All siRNAs were purchased from Dharmacon. A mix of ON-TARGETplus siRNAs directed against human LATS2 (26524) sequences GAAGUGAACCGGAAAUGC, AAUCAGAUAUUCCUUGUUG, ACACUCACCUCGCCCAAUA, GCACGCAUUUUACGAAUUC, rat LATS2 (305922) sequences GGAAAUAGCCGGCAGCGAC, UCAAUAAUGACUUGUACGA, GCAGGUUCUUCGACGACAA, ACCAGAAGGAGUCGAACUA,rat LATS1 (308265) sequences CCGAAAACCUGGCACGAUU, AUCCAAAGCCCAUCGAAUA, CAAGAAAAGUCGAUACGAA, GAGCGAUGGUAACGAGGAA, rat MOB1a(297387) sequences GGAAUGACGGUUAGGUAA, CAUACUAAAUAUAGCGUCU, AGUCAGUACUUGAUUAU, CCGAUUGACUGGUGAAUUC and rat Raptor (287871) sequencesGAGCUUGACUCCAGUUCGA, GCUAGGAACCUGAACAAAU, GCACACAGCAUGGGUGGUA, GAAUCAUGAGGUGGUAUAA. A second mix of o siRNAs directed against rat LATS2 (siGENOME; 305922)

sequencesGGAACAGCCUCAAUAAUGA, GGAAACAGCCUGCACCCUA, GAAGUUUGGACCUUAUCAA, AAGUGUGCCUUGCCUGUUAA. An ON-TARGETplus non-targeting siRNA pool from Dharmacon served as a control.

Transfections

LATS2, LATS2-KD, AMPKα1, Sestrin2, LC3 and GFP plasmids were used to overexpress these proteins in INS-1E cell. 100nM siRNAs were used for the transfection in human islets and INS-1E cells as the previously described protocol [28]. In brief, isolated islets or INS-1E cells were pre-incubated in transfection $Ca2^{+}$-KRH medium for 1h; and then lipoplexes (Lipofectamine 2000, Invitrogen)/siRNA ratio 1:20 pmol or lipoplexes/DNA ratio 2.5:1) were added to islets or INS-1E cells; after an additional 4-6h incubation,CMRL-1066 or RPMI-1640 medium containing 20% FCS was added to the transfected islets or INS-1E cells. Efficient transfection was evaluated based on GFP-positive cells analyzed by fluorescent microscopy.

Adenovirus transduction

The adenoviruses Ad-h-LATS2 expressing human LATS2 and Ad-GFP-U6-hLATS2-shRNA expressing GFP and human LATS2 shRNA were obtained from Vector Biolabs. The sequence for the shRNA of LATS2 was: CCGG-CTACTCGCCATACGCCTTTAACTCGAGTTAAAGGCGTATGGCGAGTAG-TTTTTG. Ad-LacZ or Ad-GFP-U6-shRNA were used as respective controls. For transduction, human islets were plated on ECM dishes for 24h; islets were transduced with adenoviruses (MOI 100) in CMRL-1066 medium without FCS. After 4h incubation, human islets were washed with medium and incubated for an additional 48h or treated with 22.2 mM glucose plus palmitate, or 2 ng/mL IL1-β plus 1000 U/mL IFN-γ.

Glucose-stimulated insulin secretion (GSIS)

Glucose-stimulated insulin secretion was performed by pre-incubating primary human islets in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose for 30 min, followed by KRB buffer containing 2.8 mM glucose for 1h (basal) and then an

additional 1h in KRB containing 16.7 mM glucose (stimulated), or in KRB 2.8 mM glucose with 35 mM KCl or 1 μM glibenclamide. Islets were washed with PBS and lysed with RIPA buffer to extract protein. Insulin was determined using human insulin ELISA (ALPCO Diagnostics, Salem, NH). Secreted insulin was normalized to insulin content.

TUNEL assay

Isolated human islets were cultured overnight, infected by LATS2 or LacZ control adenoviruses and fixed in Bouin's solution for 15 min before embedding in paraffin as previously described [46]. The protocol for the TUNEL assay was used according to the manufacturer's instruction (In situ Cell Death Detection Kit, TMR red; Roche) and double stained for insulin. In brief, human islet 4-μm sections were deparraffinized, rehydrated and incubated with TUNEL reagent for 1h at 37℃, followed by anti-insulin (A0546, Dako,1:100) and FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted with Vectashield with DAPI (Vector Labs). Fluorescence was analyzed using a Nikon MEA53200 (Nikon, Dusseldorf, Germany) microscope and images were acquired using NIS-Elements software (Nikon).

RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated from cultured human islets using TRIzol (Invitrogen), cDNA synthesis and quantitative RT-PCR was performed as previously described [47]. The Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, CA, USA) with TaqMan(R) Fast Universal PCR Master Mix for TaqMan assays (Applied Biosystems) were used for analysis. TaqMan(R) Gene Expression Assays were used for LATS2 (Hs00324395_m1), PDX1 (Hs00426216_m1), SLC2A2 (Hs01096905_m1), GCK (Hs01564555_m1), insulin (Hs02741908_m1), GCG(Hs01031536_s1), Nkx2.2 (Hs00159616_m1), MAFA (Hs01651425_s1), Nkx6.1 (Hs00232555_m1), NeuroD1 (HS01922995_s1), Abcc8 (Hs01093761_m1), KCNG11 (Hs00265026_s1) and PPIA(Hs99999904_m1).

Western Blot analysis

Human islets and INS-1E cells were washed twice with ice-cold PBS and lysed with RIPA lysis buffer containing Protease and Phosphatase Inhibitors (Pierce, Rockford, IL, USA). Protein concentrations were measured by the BCA protein assay (Pierce). Lysates were fractionated by NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electrically transferred into PVDF membranes. Membranes were blocked in 2.5% non-fat dry milk (CST) and 2.5% BSA (Sigma) for 1 h at room temperature and incubated overnight at 4 °C with the following antibodies: rabbit anti-LATS2 (5888),rabbit anti-LATS1 (9153), rabbit anti–Bcl2 (2870), rabbit anti–Bcl-xL (2764), mouse anti-Myc (2276), rabbit anti–cleaved caspase-3 (9664), rabbit anti-PARP (9542), rabbit anti-Cleaved PARP (9545), rabbit anti-pAMPK (2535), rabbit anti-pULK1 (5869), rabbit anti-Raptor (2280),rabbit anti-pRaptor (2083), rabbit anti-pS6K (9234), rabbit anti-pS6 (4858), rabbit anti-p4EBP1 (2855), rabbit anti-LC3B (2775), rabbit anti-tubulin (2146), rabbit anti–glyceraldehyde 3-phosphate dehydrogenase (2118) and rabbit anti-β-actin (4967) all from Cell signaling technology; rabbit anti-sestrin 2 (21346-1-AP) from proteintech; guinea pig anti-P62 (GP62-C) from PROGEN; followed by horseradish-peroxidase–linked anti-rabbit, anti-mouse or anti-guinea pig IgG secondary antibodies (Jackson). All primary antibodies were used at 1:1,000 dilution in Tris-buffered saline plus Tween-20 (TBS-T) containing 5% BSA. Membrane was developed using a chemiluminescence assay system (Pierce) and analyzed using DocITLS image acquisition 6.6a (UVP BioImaging Systems, Upland, CA, USA).

Generation of INS-1E cell with stable GFP-LC3 expression

To overexpress GFP-LC3 in INS-1E cells, cultured cells were transfected with pBABEpuro GFP-LC3 and selected with 1.5-3 μg/mL puromycin. Resistant colonies were identified by GFP under fluorescent microscopy and used for further experiments. After selection, INS-1E cell lines were maintained in culture medium containing 2 μg/ml puromycin.

Genotyping

Genomic DNA was extracted from liver, heart, spleen, kidney, hypothalamus and isolated pancreatic islets according to the manufacturer's instruction (DNeasy®Blood&Tissue Kit, QIAGEN). Genotyping was performed using the following primers: flox-F 5' CCG GAG TCA TTG CTT GTT TT 3', flox-R 5' GGA GAT CCT GGG TAC TGC AC 3', flox-F del 5' ACA TGA CAC TAC GGG GCC TAG C 3' [42]. A 300 bp band was amplified from floxed mice and 400 bpband was amplified if the LATS2 gene was deleted.

Statistical analyses

To perform statistical analysis, at least 3 independent experiments were performed for the human islets (3 different donors) and INS-1E cells, as reported in all figure legends. Data are presented as means ± SEM. Mean differences were determined by Student's t- tests. P value<0.05 was considered statistically significant.

Results

LATS2 overexpression induces β-cell death and impairs β-cell function

To investigate whether LATS2 overexpression is detrimental for β-cell survival, we overexpressed LATS2 in rat INS-1E cells and human islets through plasmid transfection or adenovirus transduction, which efficiently up-regulated LATS2 in our system (Figure 1A, B and Supplementary Figure 1). Overexpression of LATS2 itself was sufficient to induce β-cell apoptosis in both INS-1E cells and human islets, as determined by cleavage of caspase-3 and poly-(ADP-ribose) polymerase (PARP), a downstream substrate of caspase 3 (Figure 1A, B and Supplementary Figure 1). Previous data proposed a role of the mitochondrial pathway in LATS-dependent apoptotic signaling [31, 35]. Profiling expression of established mitochondrial proteins in LATS2-overexpressing islets showed a decline in anti-apoptotic Bcl-2 and Bcl-xL levels (Figure 1A, B) suggesting that LATS2-induced apoptosis proceeds via the mitochondrial-dependent pathway. Consistently, LATS2 overexpression increased the

number of TUNEL-positive β-cells in human islets confirming β-cell-specific induction of apoptosis by LATS2 hyper-activation (Figure 1C). These findings suggest that LATS2 is a strong inducer of β-cell apoptosis.

Next we checked the impact of LATS2 overexpression on insulin secretion in isolated human islets. Overexpression of LATS2 led to impairment of glucose-stimulated insulin secretion (GSIS; Figure 1D, E). To test whether the insulin secretory defect in LATS2-overexpressing islets is associated with insulin content and/or altered gene expression, we performed ELISA and RT-PCR analysis. Quantification of total insulin content as well as RT-PCR analysis of critical genes involved in glucose sensing and insulin expression reveled that LATS2 overexpression impaired GSIS in human islets (Figure 1D, E) without down-regulation neither of insulin content (data was not shown) and gene expression nor on genes involved in glucose sensing (*Slc2a2* and *GCK*), insulin transcription (*Pdx1*, *NeuroD1*, *MafA* and *Glis3*), ATP-dependent K^+ channel subunits (*Kcnj11* and *abcc8*) and key β-cell transcription factors (*Nkx2.2*, and *Nkx6.1*) (Supplementary Figure 2A,B). To gain better insight into the role of LATS2 in regulation of insulin secretion, we stimulated insulin secretion by two insulin secretagogues: KCl and glibenclamide. LATS2 overexpression significantly reduced both KCl- and glibenclamide-induced insulin secretion in human islets (Figure 1F, G), suggesting that the insulin secretory defect may occur at a step down-stream of calcium influx. Together, our data show that LATS2 impairs β-cell survival and function.

LATS2 deficiency improves β-cell survival *in vitro*

Further analyses aimed to investigate whether LATS2 inhibition can rescue β-cells from apoptosis. INS-1E cells were transfected either with siLATS1 and/or siLATS2 or control siScr and then exposed chronically to increased glucose concentrations (glucotoxicity), its combination with the free fatty acid palmitate (glucolipotoxicity), and the mixture of pro-inflammatory cytokines interleukin-1 beta (IL-1β) and interferon gamma (IFN-γ). Inhibition of endogenous LATS2 but not LATS1 activity by siRNA knockdown protected INS-1E cells from glucose-, glucose/palmitate- and

cytokine-induced apoptosis demonstrated by decreased caspase-3- and PARP-cleavage as well as increased anti-apoptotic Bcl-xL and Bcl-2 levels (Figure 2A, B and Supplementary Figure 3A, B) showing specific role of LATS2 but not LATS1 in regulation of β-cell apoptosis. A second siRNA pool to the LATS2 gene with comparable gene silencing efficiency further supported the anti-apoptotic effect of LATS2 silencing in INS-1E cells (Supplementary Figure 4A). To assess whether kinase activity of LATS2 is required for induction of apoptosis, we overexpressed a kinase dead mutant of LATS2 (LATS2-KD) in INS-1E cells. LATS2-KD overexpression profoundly reduced the levels of caspase-3 and PARP cleavage under diabetogenic conditions (Figure 2C, D). In order to further confirm the anti-apoptotic impact of LATS2 deficiency, LATS2 was silenced by siRNA-mediated transfection as well as adenoviral-mediated infection of human islets isolated from non-diabetic organ donors with Ad-shLATS2 and control viruses. Apoptosis triggered by pro-inflammatory cytokines as well as by the mixture of high glucose/palmitate was diminished by LATS2 knockdown in isolated human islets (Figure 2E, F). Our data show that loss of LATS2 activity remarkably protected β-cells and primary human islets from apoptosis under several diabetic conditions *in vitro*.

MOB1 is up-regulated under diabetic conditions and by LATS2 and is required for LATS2-induced β-cell apoptosis

Since MOB1 interacts with and activates LATS1/2 kinase activity [30, 48], we analyzed the protein expression of MOB1 in β-cells. Prolonged culture of INS-1E cells under high glucose or combination of high glucose and palmitate up-regulated MOB1 protein levels (Figure 3A). Also, overexpression of LATS2 itself increased MOB1 levels in INS-1E cells and human islets (Figure 3B, C). Reciprocally, inhibition of endogenous LATS2 but not LATS1 activity by siRNA knockdown reduced MOB1 levels under diabetic conditions suggesting LATS2-dependent regulation of MOB1 by pro-diabetic stimuli (Figure 3D and Supplementary Figure 4A, B). Also, siLATS2-induced MOB1 down-regulation was accompanied by reduced capase-3 and PARP cleavage showing positive correlation of MOB1 levels and apoptosis onset

(Figure 3D and Supplementary Figure 4A). Next, MOB1 was silenced in order to directly assess its pro-apoptotic function. Interestingly, knockdown of MOB1 antagonized the apoptotic effect of high glucose as well as high glucose/palmitate mixture in INS-1E cells (Figure 3E, F). Consistently, MOB1 knockdown reduced LATS2-induced caspase-3 cleavage (Figure 3G) indicating a major role of MOB1 in the mechanism of LATS2-induced β-cell apoptosis. These data suggest the LATS2-MOB1 axis as determinant for β-cell apoptosis under a diabetic milieu in β-cells *in vitro*.

LATS2 induces β-cell apoptosis by activating mTORC1 pathway

To identify the pathways that are regulated by LATS2 kinase in the β-cell, we have analyzed several Hippo-associated signals upon LATS2 overexpression, such as AKT [49], NOTCH [50], WNT/β-catenin [50, 51], WWOX [52] and mTOR [53]. Our immunoblot profiling revealed that mTORC1 activity was profoundly elevated by LATS2 overexpression in INS-1E cells and primary isolated human islets (Figure 4A, B). Hyper-activation of mTORC1 was demonstrated by increased phosphorylation of its downstream target S6K1 at Thr 389 (pS6K), and the direct S6K substrate ribosomal protein S6 at Ser 235/236 (pS6) (Figure 4A, B). We have recently shown that mTORC1 activity is highly up-regulated in pancreatic islets from T2D patients, animal models of T2D as well as human islets and INS-1E cells cultured under diabetes-associated glucotoxic conditions and its inhibition restored insulin secretion in T2D isolated islets (Yuan et al, 2016; *in revision*). Also, sustained hyper-activation ofmTORC1 by genetic and pharmacological tools impairs β-cell survival and controls down-stream pro-apoptotic molecules in metabolically stressed human islets and INS-1E cells as well as islets of diabetic mice (Ardestani &Maedler, unpublished data). Therefore, we further analyzed the LATS2-mTORC1 crosstalk and whether mTORC1 controls the pro-apoptotic function of LATS2 in the context of diabetes. In order to define whether LATS2 regulate mTORC1 activity under diabetic conditions in β-cells, LATS2 was first silenced and then exposed to elevated glucose or its combination with palmitate. LATS2 knockdown resulted in decreased levels of pS6K1, pS6 (Figure

4C-E) and p4EBP1 (mTORC1 readouts); this was accompanied by lower Caspase 3 and PARP cleavage (Figure 4C) and provides direct evidence of mTORC1 regulation by LATS2, which was up-regulated upon exposure to a diabetic milieu. To establish whether mTORC1-S6K1 signaling inhibition is sufficient to block pro-apoptotic function of LATS2 in β-cells, we targeted mTORC1 in INS-1E cells and human islets by the use of selective inhibitors against mTOR (rapamycin) and S6K1 (PF-4708671; S6K1i) (Figure 4F, G). Western blot analysis of LATS2-overexpressing INS-1E cells and human islets treated with rapamycin or S6K1i revealed the full blockade of mTORC1 (represented by pS6) together with counteracting of apoptosis, as observed by Casp3 or PARP cleavage (Figure 4F, G). In line with this observation, selective inhibition of endogenous mTORC1 by siRNA-mediated silencing of Raptor, mTORC1's critical subunit, also reduced mTORC1 signaling efficiently and substantially protected INS-1E cells from LATS2-induced apoptosis (Figure 4H) further corroborating the hyper-activated mTORC1 as down-stream player of LATS2 in the context of β-cell apoptosis. Altogether, these data suggest LATS2-induced β-cell apoptosis is mediated by mTORC1 activation.

LATS2 inducesmTORC1 activation and β-cell apoptosis by inhibition of AMPK

As nutrient-sensing protein, AMP-activated protein kinase (AMPK) is an established negative regulator of mTORC1 signaling [54] and is suppressed in nutrient-enriched conditions in pancreatic β-cells [55], we hypothesized that LATS2 might regulate mTORC1 by AMPK. AMPK activity was enhanced by overexpression of AMPK catalytic subunit alpha (α) (activated AMPK) in INS-1E cells and confirmed by up-regulation of AMPK-α phosphorylation at activation loop Thr 172 (pAMPK) and its down-stream substrates including Phospho-Acetyl-CoA Carboxylase (ACC) at Ser79 (pACC), UNC-51-like kinase 1 (ULK1) at Ser 555 (pULK1) and Raptor at Ser792 (pRaptor)(Figure 5A).While LATS2 overexpression decreased the phosphorylation of AMPK and its target phosphorylation of ACC, LATS2-induced mTORC1 activation as well as apoptosis was reduced by activated AMPK overexpression (Figure 5A). Sestrin-2 (SESN2) is an endogenous activator of AMPK and can inhibit mTORC1

through AMPK-dependent and -independent mechanism [45, 56]. Overexpression of SESN2 antagonized LATS2-induced mTORC1 up-regulation and reduced induction of Caspase 3 and PARP cleavages (Figure 5B). The *in vitro* model of starvation-induced AMPK activation has been applied in order to further confirm such inhibitory action from LATS2 to AMPK. While starvation rapidly activated AMPK and its down-stream targets (pULK1/pACC/pRaptor) in a time-dependent manner in LacZ-infected control INS-1E cells, LATS2 overexpression profoundly decreased the extent of AMPK activation and its related signals especially at prolonged exposure of 8h (Figure 5C). Our findings indicate that LATS2 might activate mTORC1 and induce β-cell apoptosis by inhibition of AMPK suggesting that AMPK might be intermediate signaling pathway interconnecting LATS2 to mTORC1 and β-cell apoptosis.

LATS2 regulates defective autophagy-induced β-cell apoptosis

Autophagy is an intracellular self-degradative catabolic process and plays a critical role for cell viability and homoeostasis [57]. Defective autophagy is a hallmark of β-cell failure in T2D [58-61]. AMPK and mTORC1 reciprocally control the autophagic flux through different mechanism including direct phosphorylation and regulation of ULK1 activity [62]. In order to investigate whether autophagy participates in LATS2-induced β-cell apoptosis, we treated INS-1E cells and human islets with two different autophagy inhibitors: BafilomycinA1 (Baf) and Chloroquine (CQ). Suppression of autophagic flux by Baf as well as CQ was sufficient to trigger apoptosis, which was exacerbated by LATS2 overexpression in INS-1E (Figure 6A, B) and stable GFP-LC3 expressing INS-1E cells (Supplementary Figure 5A). Consistently, LATS2-overexpressing human islets exhibited higher amount of cleaved Caspase 3 and LC3-BII as well as p62 accumulation (Figure 6C) showing further potentiation of LATS2 induced cell death by Baf-mediated impaired autophagy (represented by LC3-BII and p62 levels). Conversely, Baf- or CQ-induced β-cell apoptosis was greatly decreased by LATS2 silencing in INS-1E and GFP-LC3 expressing INS-1E cells as well as human islets (Figure 6D-F and Supplementary Figure 5B). Also, LATS2 deficiency reduced Baf- or CQ-induced LC3-BII and P62

accumulation in human islets (Figure 6F). Interestingly, we found that endogenous LATS2 protein levels were increased upon Baf or CQ treatments in INS-1E cells and human islets (Figure 6D-F) proposing LATS2 as potential substrate for autophagy-mediated degradation. These data suggest the existence of a mutual regulatory axis between LATS2 and autophagy to fine-tune the β-cell apoptosis program. On the one hand, LATS2 controlled defective autophagy-mediated apoptosis; on the other hand, autophagy regulated endogenous LATS2 protein levels for balanced survival/apoptosis.

LATS2-deletion protects from MLD-STZ-induced diabetes

As LATS2 depletion protected from β-cell apoptosis under multiple diabetic conditions *in vitro*, we hypothesized that LATS2 deficiency may protect against diabetes development *in vivo*. To test this hypothesis, we generated β-cell-specific LATS2 knockout mice (β-LATS2$^{-/-}$) by the Cre-lox system. We crossed LATS2 floxed (LATS2$^{fl/fl}$) mice with the β-cell-specific Cre transgenic line driven by the rat insulin promoter (Rip-Cre). Rip-Cre-mediated specific deletion of LATS2 gene in pancreatic β-cell was confirmed in isolated islets from β-LATS2$^{-/-}$ and LATS2$^{fl/fl}$mice by western blotting (Supplementary 6A). Rip-Cre has been reported to delete genes in β-cells and also in a poorly characterized population of hypothalamic neurons [63]. To test the specificity of LATS2 gene deletion, DNA was isolated from liver, spleen, kidney, heart, hypothalamus and pancreatic islets of β-LATS2$^{-/-}$ and LATS2$^{fl/fl}$ mice. PCR analysis of genomic DNA prepared from LATS2$^{fl/fl}$ and β-LATS2$^{-/-}$mice demonstrated that Cre-mediated LATS2 deletion was islet specific with no leakage in the hypothalamus or any other tested tissues (Supplementary Figure 6B). β-LATS2$^{-/-}$ mice were viable, fertile and showed no difference in food intake and body weight compared to LATS2$^{fl/fl}$ mice, or LoxP-negative mice (Rip-Cre mice; data not shown). To assess whether β-LATS2$^{-/-}$mice might protect against β-cell injury and diabetes, we induced diabetes by multiple-low dose streptozotocin (MLD-STZ) injection inβ-LATS2$^{-/-}$, LATS2$^{fl/fl}$ and Rip-Cre mice. While MLD-STZ injection induced progressive hyperglycemia and severely impaired glucose tolerance in LATS2$^{fl/fl}$ and

Rip-Cre mice, blood glucose levels were significantly reduced and glucose tolerance improvedin the β-LATS2$^{-/-}$ mice at all time points during the intraperitoneal glucose tolerance test (Figure 7A, B). Also, glucose-induced insulin secretion was fully blunted and insulin-to-glucose ratio was decreased in MLD-STZ-treated LATS2$^{fl/fl}$ and Rip-Cre mice, which were significantly restored in β-LATS2$^{-/-}$ mice (Figure 7C-E). Our data show that β-cell-specific ablation of LATS2 diminished progressive hyperglycemia and improved glucose tolerance and insulin secretion in MLD-STZ mouse model of β-cell destruction and diabetes.

Discussion

Our work shows that LATS2 acts as a pro-apoptotic molecule and participates in different signaling pathways which leads to β-cell failure through increased β-cell apoptosis and impaired β-cell function. We identified AMPK-mTORC1-autophagy pathway as a downstream signaling of LATS2-induced β-cell apoptosis (Supplementary Figure 7); loss of LATS2 resulted in resistance to apoptosis induced by diabetogenic conditions *in vitro* and improved glycemia and insulin secretion in the MLD-STZ mouse model *in vivo*.

LATS2 plays a crucial role the in regulation of proliferation and apoptosis. While LATS2 overexpression induces apoptosis in various cells [31, 35, 64], loss of LATS2 activity by silencing or overexpression of dominant negative form of LATS2 (DN-LATS2) reduces apoptosis [29, 33]. Consistent with the role of LATS2 as an established pro-apoptotic kinase in other cell types, we show here that overexpression of LATS2 alone was sufficient to trigger β-cell apoptosis in an established β-cell line and in primary human islets *in vitro*. In contrast, loss of LATS2 in β-cells leads to protection from apoptosis in INS-1E cells and primary human islets under diabetic conditions including pro-inflammatory cytokines, gluco- and lipo-toxicity. An *in vivo* MLD-STZ study proved that β-cell-specific LATS2 deletion protected mice from hyperglycemia and development of diabetes by improving glucose tolerance, and insulin secretion. Thus, LATS2 is a critical regulator of β-cell survival in the pancreas *in vivo*.

Our findings show that LATS2 overexpression impaired insulin secretion in human isolated islets without down-regulating insulin gene expression and content. Also, LATS2 did not down-regulate genes involved in glucose sensing and insulin transcription. Notably, LATS2 also impaired stimulated insulin secretion in conjunction with glibenclamide (closure of the ATP-dependent K^+ channel) as well as KCl (depolarization of β-cells and opening of voltage-gated Ca^{2+} channels), suggesting its actions are downstream of the ATP-dependent potassium channel. β-cell calcium (Ca^{2+}) signaling is a critical regulator of insulin secretion [65]. The mechanisms underlying the impaired insulin secretion by LATS2 need further detailed elucidation, i.e. the expression of the voltage-dependent Ca^{2+} channels in human β-cells including the P/Q-type channels (CACNA1A), L-type Ca^{2+} channels (CACNA1C and CACNA1D) and the Ca^{2+} channel beta-2 subunit (CACNB2). To also determine whether LATS2 alters the concentration of cytosolic free Ca^{2+} $[Ca^{2+}]_i$ as a mechanism to reduce insulin secretion, islets'$[Ca^{2+}]_I$ concentrations upon LATS2 overexpression in response to glucose, KCl and other stimulus should be analyzed in future studies.

The mTORC1 pathway has a critical role in the regulation of cell growth and metabolism. Recent studies showed that mTORC1 activity is elevated in the liver, fat, muscle and pancreatic islets of *db/db* and high fat diet mice [66, 67]. In addition, mTORC1 is activated in the kidney of obese individuals and patients with type 2 diabetes by hyperinsulinemia and hyperglycemia [68]. As discussed in manuscript-part III of this thesis, prolonged activation of mTORC1 in β-cells leads to a metabolic shift from hypoglycemia and hyperinsulinemia to hyperglycemia and hypoinsulinemia as a result of multiple cellular metabolic and non-metabolic abnormalities such ER and oxidative stresses and defective mitochondrial function which leads to severe β-cell destruction [67, 69]. In line with the deleterious chronic hyper-activation of mTORC1 signaling in context of over-nutrition, S6K1-deficient mice are protected from diet- and age-induced obesity [66]. Consistently, we show that mTORC1 activity is up-regulated in islets of T2D patients and metabolically stressed β-cells and human islets (Yuan et al, 2016, in revision) but underlying up-stream mechanisms regulating mTORC1 hyper-activation in the context of

diabetes were not clear so far. In this study, we identified the kinase LATS2 as principal regulator of mTORC1 activity in β-cells; its deficiency ameliorates mTORC1 hyper-activation and β-cell apoptosis under T2D-relevant stressors and its overexpression copies cellular abnormalities seen in diabetic islets such as highly up-regulated mTORC1 and increased apoptosis. Also, genetic and pharmacological targeting of mTORC1 demonstrated the causative and deleterious activation of mTORC1 in LATS2-overexpressed β-cells and human islets.

AMPK is an energy sensor and activated by nutrient-depleted conditions. AMPK expression and activity decrease in the STZ-induced T1D model [70]. Moreover, AMPK is dysregulated in the development of insulin resistance and T2D. Consequently, AMPK activation is able to improve insulin sensitivity and metabolic health [71, 72]. Such activated AMPK can inhibit mTORC1 through two separate pathways: phosphorylation and activation of tuberous sclerosis complex 2 (TSC2) [54], a negative regulator of mTORC1; and phosphorylation of Raptor at Ser863 leading to mTORC1 de-stabilization and inactivation [73]. LATS2, AMPK and mTORC1 are components of three interconnected stress- or energy-triggered signaling pathways, which functionally regulate each other. Activated LATS2 itself up-regulates mTORC1 function by AMPK suppression in the β-cells, indicating the existence of a complex interplay and potential linear or non-linear crosstalk among these three pathways. Our data show that LATS2-induced AMPK inactivation triggers higher mTORC1 activity and subsequent β-cell apoptosis. Conversely, direct activation of AMPK either by overexpression of its catalytic subunit or introducing SESN2, an endogenous AMPK activator, reversed LATS2-induced mTORC1 hyper-activation and β-cell death. Our results suggest that LATS2, AMPK and mTORC1 constitute a stress-sensitive survival pathway. Under acute stress conditions, AMPK promoted β-cell survival by inhibiting mTORC1 and restoring protective-autophagy mechanism, but prolonged stress activated LATS2 leading to AMPK inactivation, mTORC1 hyper-activation, defective autophagy and subsequent β-cell apoptosis. This antagonism between AMPK and mTORC1 suggests that the outcome of the mutual regulation of both pathways under conditions of increased β-cell stress and demand in a T2D environment is probably

determined by the extent and duration of the activated LATS2. In this context, aberrant LATS2 activity triggered by diabetic stimuli may shift the balance towards the impairment of AMPK, resulting in mTORC1-dependent defective autophagic flux and β-cell apoptosis. To make the story more complicated, our preliminary experiments revealed autophagy-dependent regulation of endogenous LATS2 levels suggesting the existence of multicomponent cellular loops where autophagy as down-stream target of LATS2 (through AMPK-mTORC1) may function as up-stream signal to regulate LATS2 activity by controlling its degradation. However, further mechanistic studies and complementary cellular and animal models are required for more comprehensive understanding of such complex crosstalk at basal- and disease-states.

In summary, we have used a multi-model approach to uncover the deleterious role of LATS2 on β-cell survival and function. Our results identified LATS2 as a novel pro-apoptotic kinase whose over-expression led to β-cell death and impaired β-cell function and its depletion improvedβ-cell survival. Blocking of LATS2 may be a successful strategy to improve β-cell survival and function in diabetes.

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Legends

Figure 1. LATS2 induces β-cell death and impairs β-cell function. (A) Representative Western blot of INS-1E cells transfected with LATS2 or GFP control plasmids for 48h. (B-G) Human islets were transduced with LacZ control or LATS2 adenoviruses for 48h. (B) Representative Western blot analysis. (C) Double staining for TUNEL (red) and insulin (green). Pooled TUNEL analysis was from 5 independent experiments from 5 different human islets preparations. (D) Insulin secretion during 1 h incubation with 2.8 mM (basal) and 16.7 mM (stimulated) glucose, normalized to insulin content. (E) Insulin stimulatory index denotes the ratio of stimulated (16.7 mM glucoses) and basal (2.8 mM glucose). (D, E) Pooled data from 7 independent experiments from 7 different human islets preparations. (F, G) Insulin secretion during 1 h incubation with 2.8 mM (Basal), 2.8 mM glucose plus 35 mM KCl and 2.8mM glucose plus 1 μM glibenclamide. Pooled data from 4 independent experiments from 4 different human islets preparations. All western blots show representative results from at least 3 independent experiments from 3 different donors (human islets). Data are expressed as means ± SEM. *$p<0.05$ Ad-LATS2 compared to Ad-LacZ control transduced islets.

Figure 2. LATS2 deficiency improves β-cell survival *in vitro.* (A, B) Representative Western blots of INS-1E cells transfected with LATS2 siRNA or control siScr and treated with 22.2 mM glucose, 22.2 mM glucose plus 0.5 mM palmitate, or 2 ng/mL IL1β (IL) plus 1000U/mL IFNγ (IF) for 48 h. (C, D) Representative Western blots of INS-1E cells transfected with GFP or kinase-dead form of LATS2 (LATS2-KD) and then treated with (C) 22.2 mM glucose or (D) mixture of IL/IF for 48 h. (E, F) Representative Western blots of human islets transfected with siLATS2 or transduced with Ad-hShLATS2 and treated with (E) 22.2 mM glucose plus 0.5 mM palmitate or (F) mixture of IL/IF for 72 h. Western blots show representative results from at least 3 independent experiments (A-D) in INS-1E cells and from 3 (E) and 4 (F) different human islets donors.

Figure 3. MOB1 regulates β-cell apoptosis. (A) Representative Western blots of INS-1E cells treated with 22.2 mM glucose or 22.mM glucose plus 0.5 mM palmitate for 48 h. (B,C) Representative Western blots of INS-1E cells (B) and human islets (C) transduced with LacZ control or LATS2 adenoviruses for 48 h. (D) Representative Western blots of INS-1E cells transfected with LATS2 siRNA or control siScr and treated with 22.2 mM glucose for 48 h. (E, F) Representative Western blots of INS-1E cells transfected with MOB1 siRNA or control siScr and treated with 22.2 mM glucose (E) or 22.mM glucose plus 0.5 mM paltimate (F) for 48 h. (G) Representative Western blot of INS-1E cells transfected with SiMOB1 or siScr and transduced with Ad-LacZ or Ad-LATS2 for 48h. All western blots show representative results from at least 3 independent experiments.

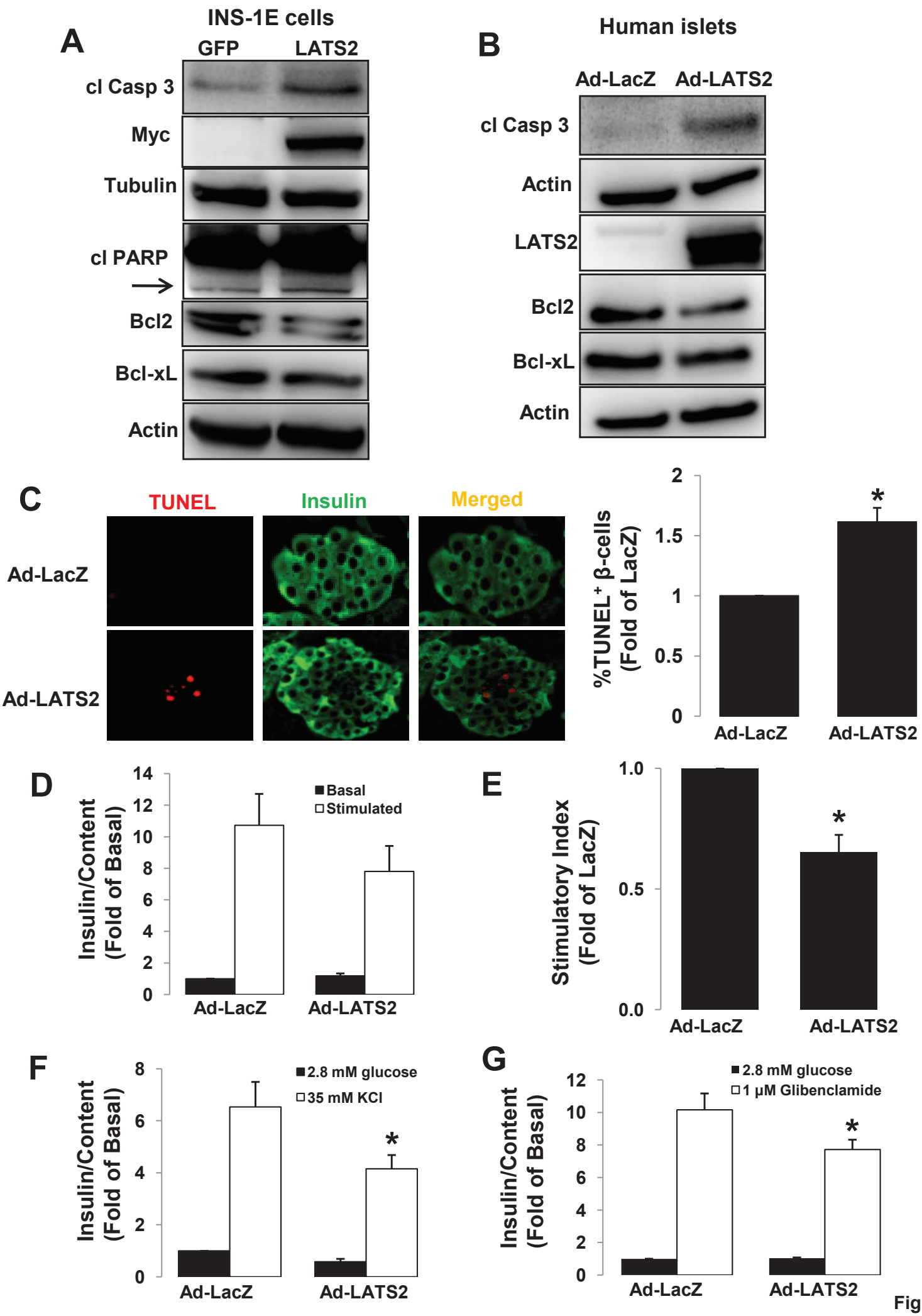
Figure 4. LATS2 induces β-cell apoptosis by activating mTORC1 pathway. (A, B) Representative Western blots of INS-1E cells (A) and human islets (B) transduced with LacZ control or LATS2 adenoviruses for 48 h. (C,D) Representative Western blots of INS-1E cells transfected with LATS2 siRNA or control siScr and treated with the (C) 22.2 mM glucose, or (D) 22.2 mM glucose plus 0.5 mM palmitate. (E) Representative Western blot of human islets transduced with Ad-hShLATS2 and treated with 22.2 mM glucose plus 0.5 mM palmitate for 72 h. (F, G) Representative Western blots of INS-1E cells (F) and human islets (G) transduced with LacZ control or LATS2 adenoviruses for 24 h and then exposed to 100 nM Rapamycin or 10 μM S6K1 inhibitor for additional 24 h. (H) Representative Western blot of INS-1E cells transfected with siRaptor or siScr and then transduced with Ad-LacZ or Ad-LATS2 for 48 h. All western blots show representative results from at least 3 independent experiments from 3 different donors (human islets).

Figure 5. LATS2-AMPK crosstalk. (A) Representative Western blot of INS-1E cells transfected with AMPK catalytic subunit alpha (α) (activated AMPK) or GFP and then transduced with Ad-LacZ or Ad-LATS2 for 48h. (B) Representative Western blot of INS-1E cells transfected with GFP or Sestrin-2 (SESN2) and then transduced with Ad-LacZ or Ad-LATS2 for 48h. (C) Representative Western blot of INS-1E cells transduced with Ad-LacZ or Ad-LATS2 for 48h and then starved with HBSS media for 4 and 8h. All western blots show representative results from at least 3 independent experiments.

Figure 6. LATS2 regulates defective autophagy-induced β-cell apoptosis. Representative Western blots of INS-1E cells (A, B) and human islets (C) transduced with Ad-LacZ or Ad-LATS2 and treated with 20 nM Bafilomycin A1 (Baf) or 50 μM Chloroquine (CQ) for 4h. (D, E) Representative Western blots of INS-1E cells transfected with siLATS2 or siScr and treated with Baf (D) or CQ (E) for 4h. (F) Representative Western blot of human islets transduced with Ad-hShLATS2 or Ad-shScr and treated with Baf for 4h. Western blots show representative results from at least 3 independent experiments (A, B, D, E) and from 1 (C) and 3 (F) different human islets donors.

Figure 7. LATS2-deletion protects from STZ-induced diabetes. β-LATS2$^{-/-}$ mice with specific deletion in the β-cells using the Cre-*lox* system (*n*=14), RIP-Cre (*n*=7) and LATS2$^{fl/fl}$ controls (*n*=10) injected with 40 mg per kg body weight streptozotocin (STZ) or saline (β-LATS2$^{-/-}$*n*=4; LATS2$^{fl/fl}$*n*=5) for five consecutive days. (A) Random fed blood glucose measurements after first saline or STZ injection (40 mg per kg body weight for 5 consecutive days) (day 0) over 21 days and (B) i.p. glucose tolerance test (GTT) at day 19 in β-LATS2$^{-/-}$, Rip-Cre and LATS2$^{fl/fl}$ mice. (C) Insulin secretion during an i.p.GTTmeasured before (0min) and 15 min after glucose injection and expressed (D) as ratio of secreted insulin at 15 min to that secreted at 0 min (stimulatory index). (E) Ratio of secreted insulin and glucose calculated at fed state. Data are expressed

as means ± SEM. *p<0.05 β-LATS2$^{-/-}$-STZ compared to RIP-Cre-STZ or LATS2$^{fl/fl}$-STZ mice.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Basal
Stimulated
Ad-LacZ
Ad-LATS2
E
Stimulatory Index (Fold of LacZ)
1.0
0.5
0.0
*
Ad-LacZ
Ad-LATS2
F
Insulin/Content (Fold of Basal)
8
6
4
2
0
2.8 mM glucose
35 mM KCl
*
Ad-LacZ
Ad-LATS2
G
Insulin/Content (Fold of Basal)
12
10
8
6
4
2
0
2.8 mM glucose
1 μM Glibenclamide
*
Ad-LacZ
Ad-LATS2

Figure 1

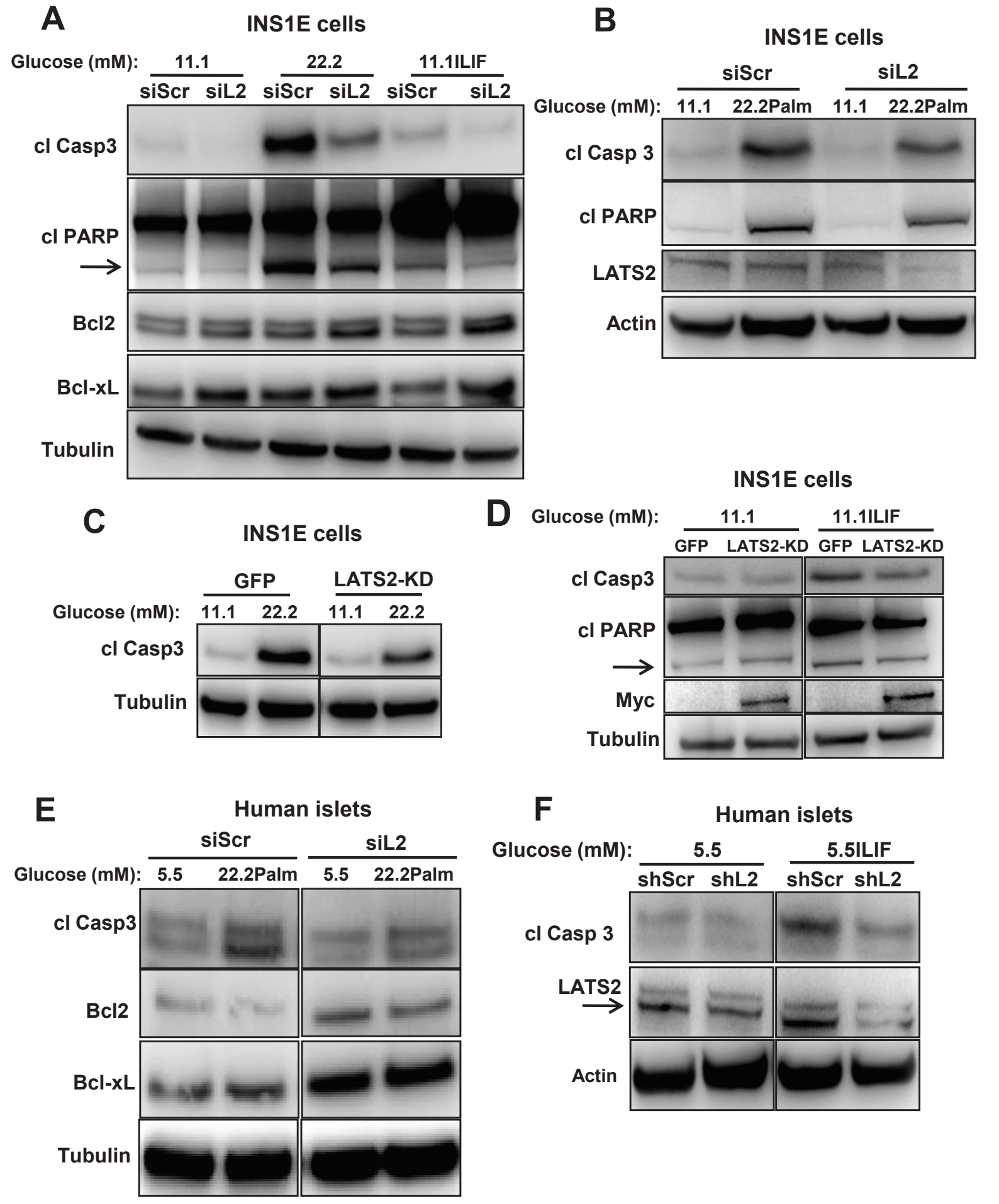

A
INS1E cells
Glucose (mM):
11.1
22.2
11.1ILIF
siScr
siL2
cl Casp3
cl PARP
Bcl2
Bcl-xL
Tubulin
B
INS1E cells
siScr
siL2
Glucose (mM):
11.1
22.2Palm
cl Casp 3
cl PARP
LATS2
Actin
C
INS1E cells
GFP
LATS2-KD
Glucose (mM):
11.1
22.2
cl Casp3
Tubulin
D
INS1E cells
Glucose (mM):
11.1
11.1ILIF
GFP
LATS2-KD
cl Casp3
cl PARP
Myc
Tubulin
E
Human islets
siScr
siL2
Glucose (mM):
5.5
22.2Palm
cl Casp3
Bcl2
Bcl-xL
Tubulin
F
Human islets
Glucose (mM):
5.5
5.5ILIF
shScr
shL2
cl Casp 3
LATS2
Actin

Figure 2

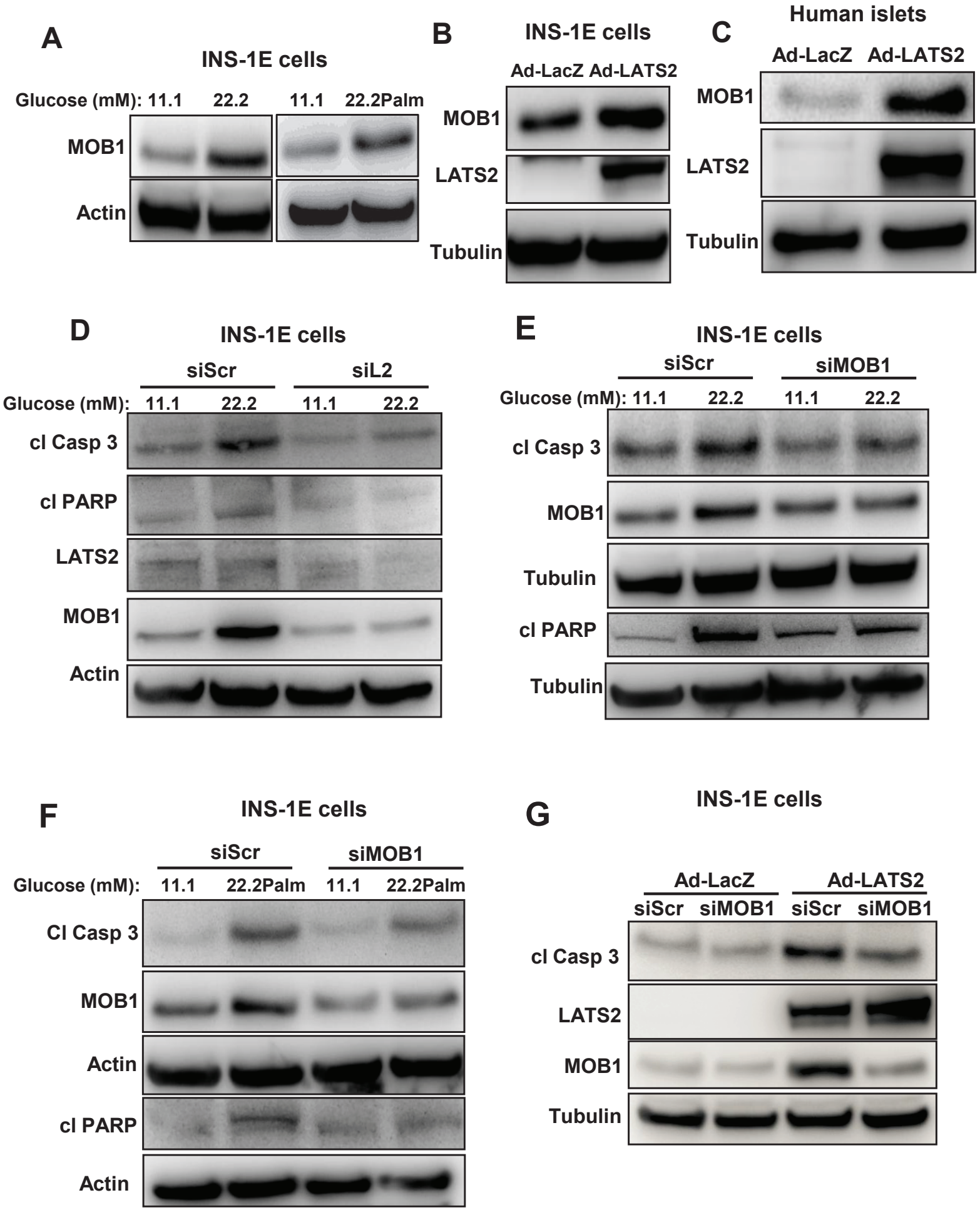

A
INS-1E cells
Glucose (mM): 11.1 22.2 11.1 22.2Palm
MOB1
Actin
B
INS-1E cells
Ad-LacZ Ad-LATS2
MOB1
LATS2
Tubulin
C
Human islets
Ad-LacZ Ad-LATS2
MOB1
LATS2
Tubulin
D
INS-1E cells
siScr siL2
Glucose (mM): 11.1 22.2 11.1 22.2
cl Casp 3
cl PARP
LATS2
MOB1
Actin
E
INS-1E cells
siScr siMOB1
Glucose (mM): 11.1 22.2 11.1 22.2
cl Casp 3
MOB1
Tubulin
cl PARP
Tubulin
F
INS-1E cells
siScr siMOB1
Glucose (mM): 11.1 22.2Palm 11.1 22.2Palm
Cl Casp 3
MOB1
Actin
cl PARP
Actin
G
INS-1E cells
Ad-LacZ Ad-LATS2
siScr siMOB1 siScr siMOB1
cl Casp 3
LATS2
MOB1
Tubulin

Figure 3

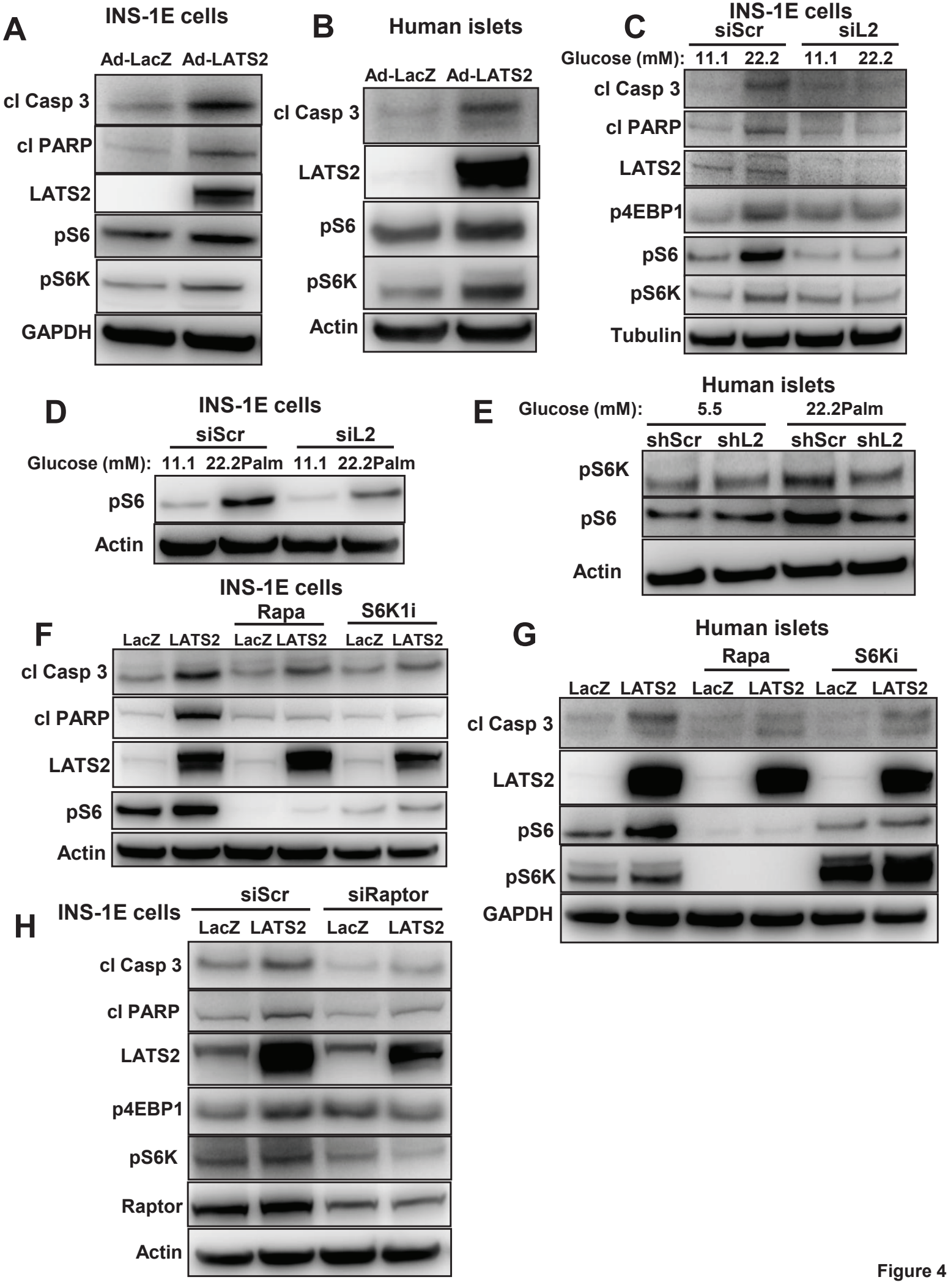

A
INS-1E cells
Ad-LacZ
Ad-LATS2
cl Casp 3
cl PARP
LATS2
pS6
pS6K
GAPDH
B
Human islets
Ad-LacZ
Ad-LATS2
cl Casp 3
LATS2
pS6
pS6K
Actin
C
INS-1E cells
siScr
siL2
Glucose (mM):
11.1
22.2
11.1
22.2
cl Casp 3
cl PARP
LATS2
p4EBP1
pS6
pS6K
Tubulin
D
INS-1E cells
siScr
siL2
Glucose (mM):
11.1
22.2Palm
11.1
22.2Palm
pS6
Actin
E
Human islets
Glucose (mM):
5.5
22.2Palm
shScr
shL2
shScr
shL2
pS6K
pS6
Actin
F
INS-1E cells
Rapa
S6K1i
LacZ
LATS2
LacZ
LATS2
LacZ
LATS2
cl Casp 3
cl PARP
LATS2
pS6
Actin
G
Human islets
Rapa
S6Ki
LacZ
LATS2
LacZ
LATS2
LacZ
LATS2
cl Casp 3
LATS2
pS6
pS6K
GAPDH
H
INS-1E cells
siScr
siRaptor
LacZ
LATS2
LacZ
LATS2
cl Casp 3
cl PARP
LATS2
p4EBP1
pS6K
Raptor
Actin

Figure 4

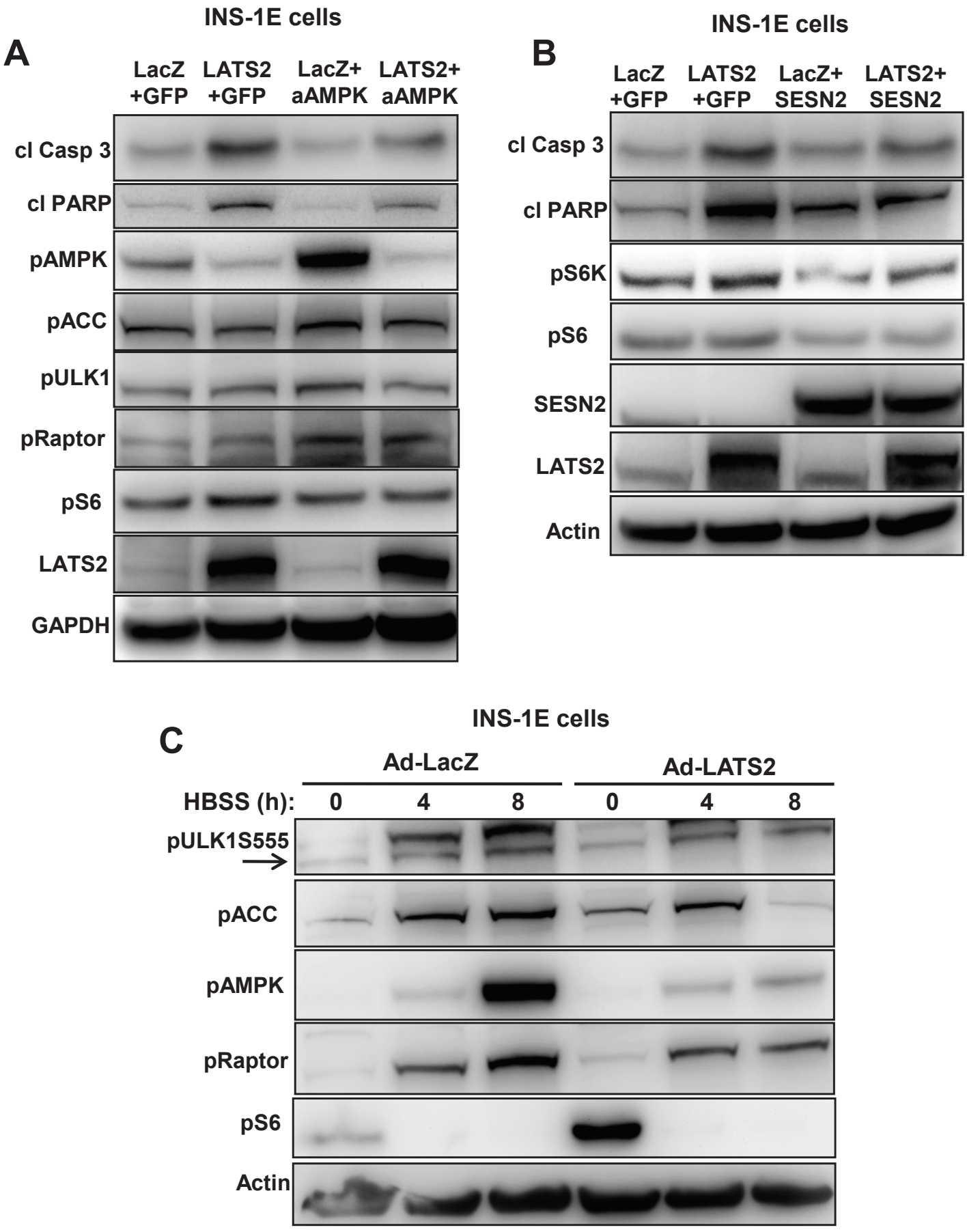

A
INS-1E cells
LacZ +GFP
LATS2 +GFP
LacZ+ aAMPK
LATS2+ aAMPK
cl Casp 3
cl PARP
pAMPK
pACC
pULK1
pRaptor
pS6
LATS2
GAPDH
B
INS-1E cells
LacZ +GFP
LATS2 +GFP
LacZ+ SESN2
LATS2+ SESN2
cl Casp 3
cl PARP
pS6K
pS6
SESN2
LATS2
Actin
C
INS-1E cells
Ad-LacZ
Ad-LATS2
HBSS (h):
0
4
8
0
4
8
pULK1S555
pACC
pAMPK
pRaptor
pS6
Actin

Figure 5

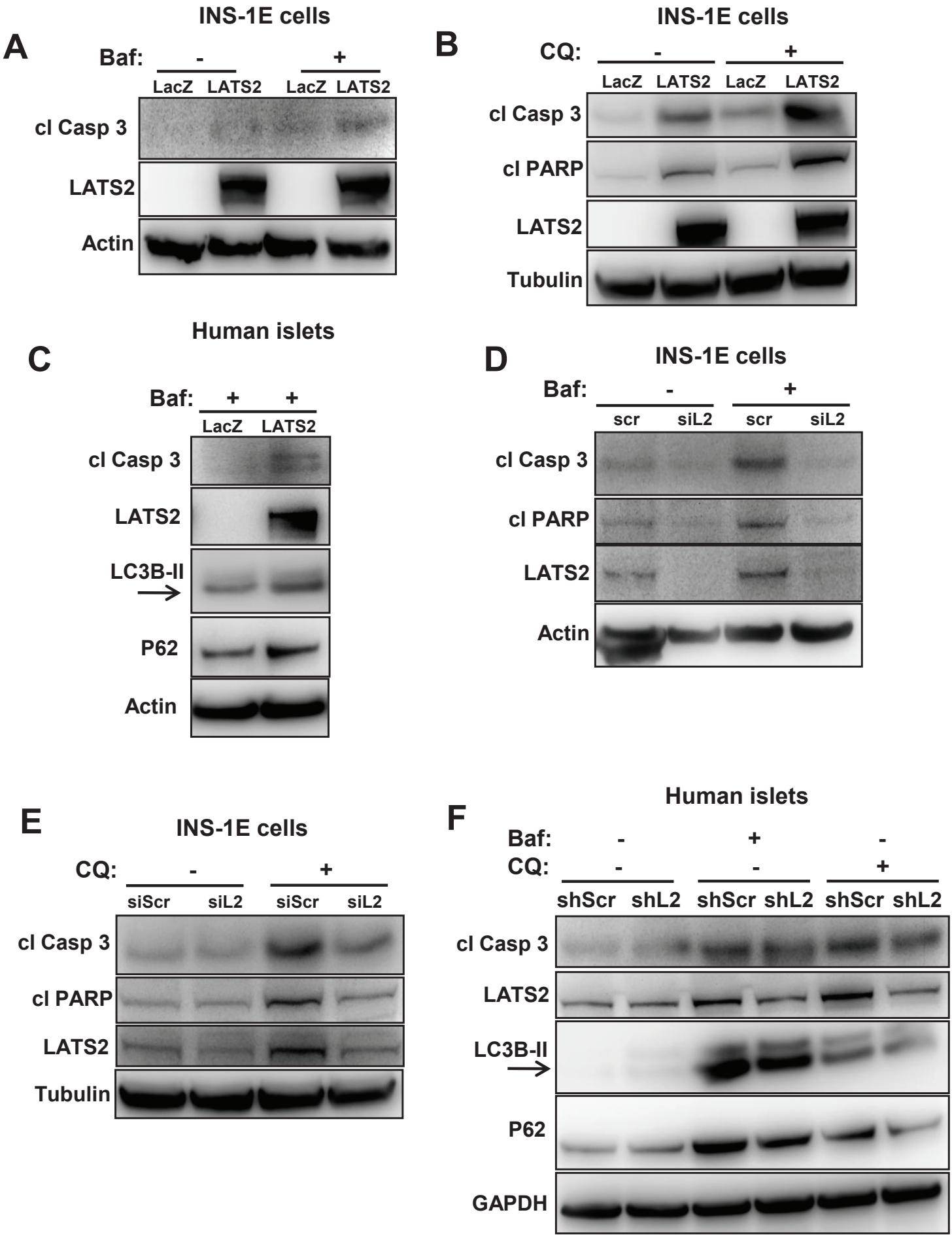

A
INS-1E cells
Baf: - +
LacZ LATS2 LacZ LATS2
cl Casp 3
LATS2
Actin
B
INS-1E cells
CQ: - +
LacZ LATS2 LacZ LATS2
cl Casp 3
cl PARP
LATS2
Tubulin
C
Human islets
Baf: + +
LacZ LATS2
cl Casp 3
LATS2
LC3B-II
P62
Actin
D
INS-1E cells
Baf: - +
scr siL2 scr siL2
cl Casp 3
cl PARP
LATS2
Actin
E
INS-1E cells
CQ: - +
siScr siL2 siScr siL2
cl Casp 3
cl PARP
LATS2
Tubulin
F
Human islets
Baf: - + -
CQ: - - +
shScr shL2 shScr shL2 shScr shL2
cl Casp 3
LATS2
LC3B-II
P62
GAPDH

Figure 6

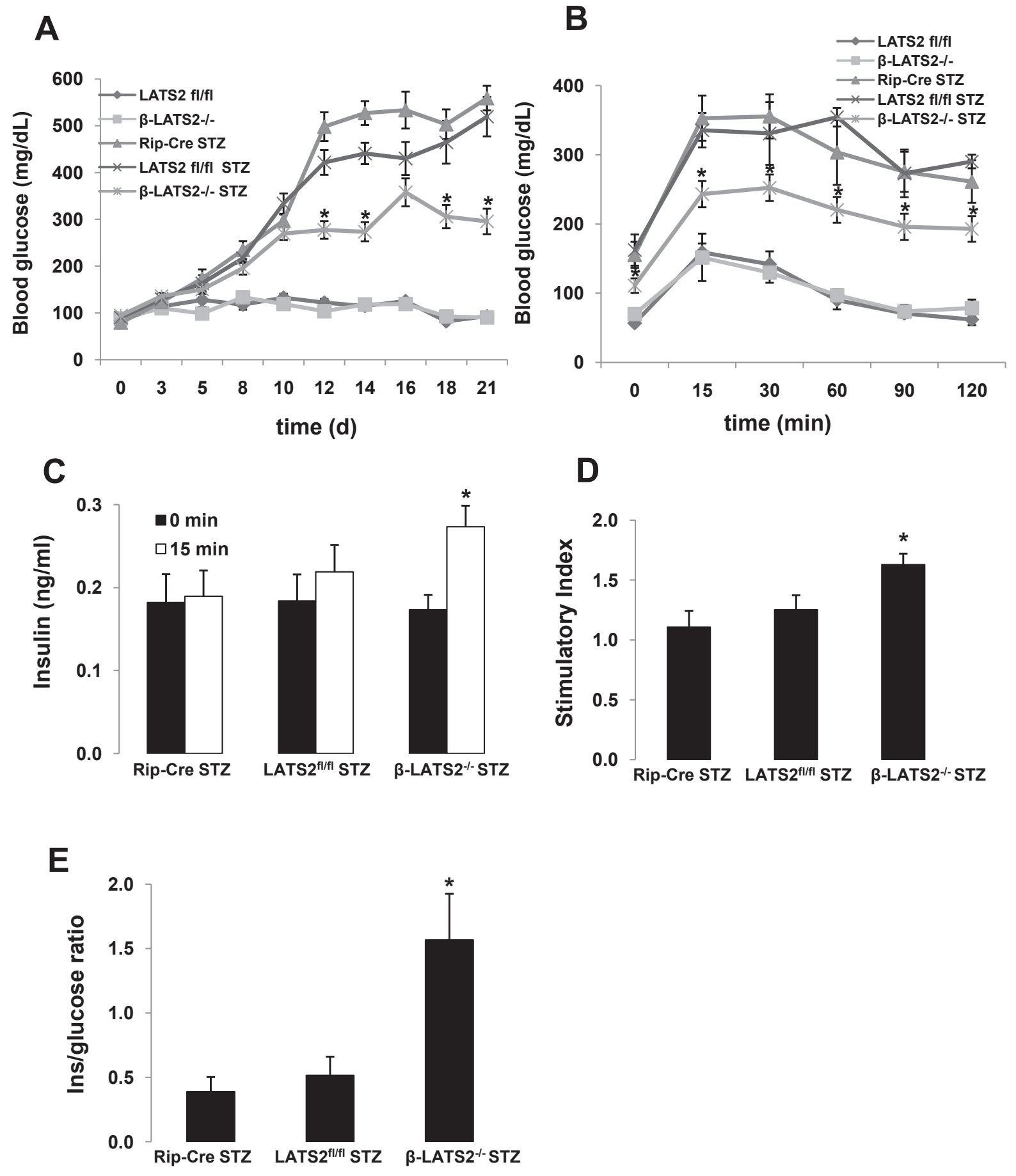
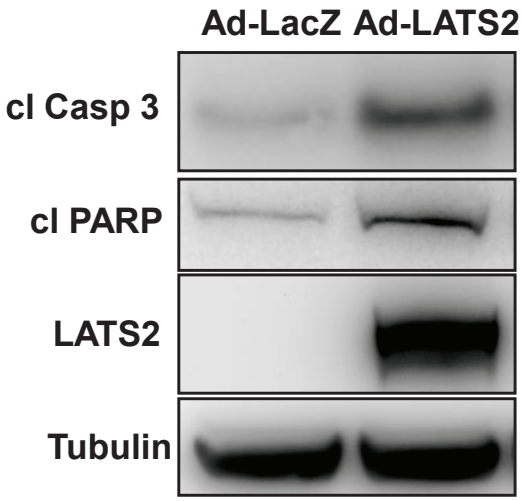
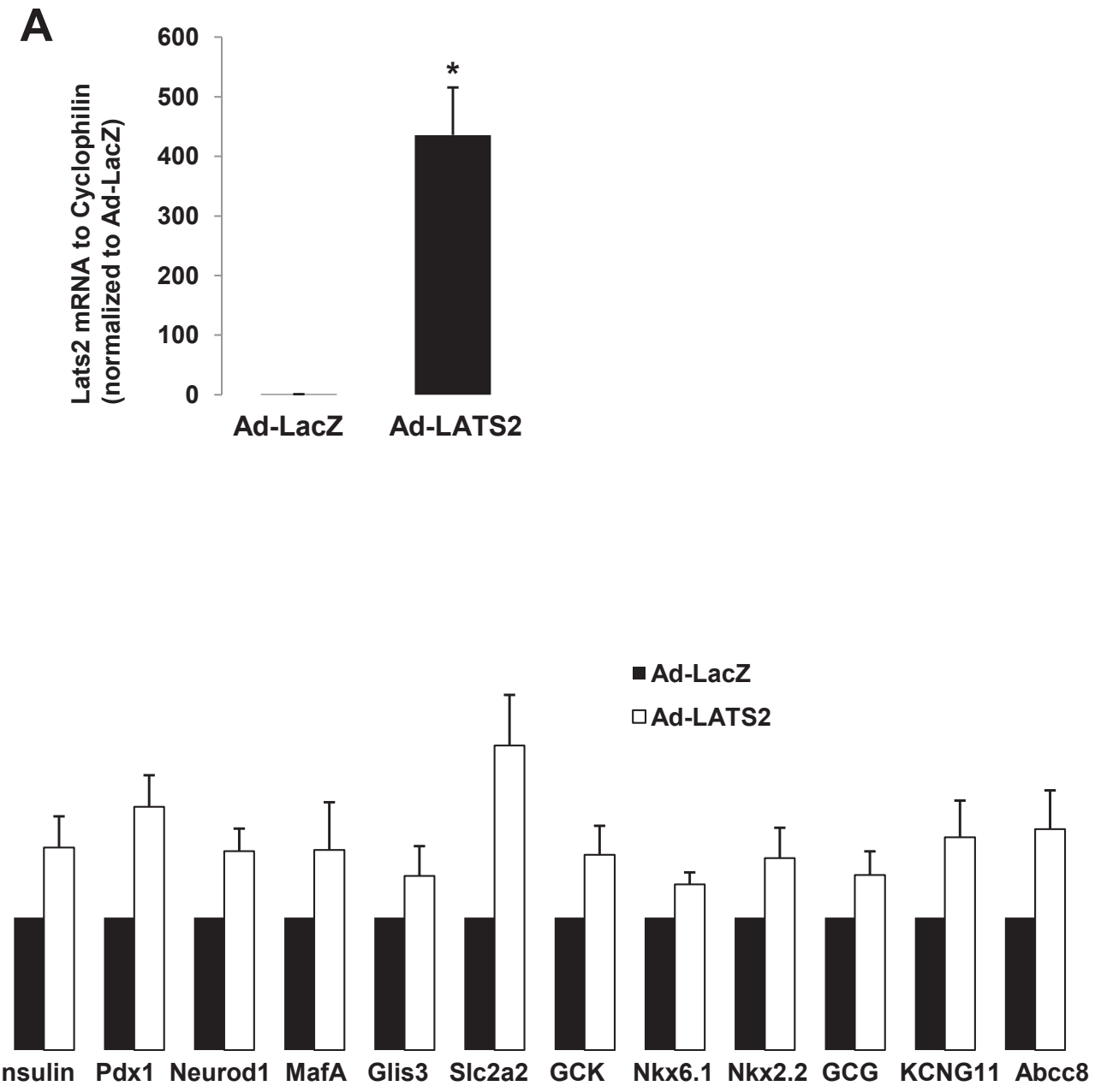

A
Blood glucose (mg/dL)
600
500
400
300
200
100
0
LATS2 fl/fl
β-LATS2-/-
Rip-Cre STZ
LATS2 fl/fl STZ
β-LATS2-/- STZ
0 3 5 8 10 12 14 16 18 21
time (d)
B
Blood glucose (mg/dL)
400
300
200
100
0
LATS2 fl/fl
β-LATS2-/-
Rip-Cre STZ
LATS2 fl/fl STZ
β-LATS2-/- STZ
0 15 30 60 90 120
time (min)
C
Insulin (ng/ml)
0.3
0.2
0.1
0.0
0 min
15 min
Rip-Cre STZ
LATS2fl/fl STZ
β-LATS2-/- STZ
D
Stimulatory Index
2.0
1.5
1.0
0.5
0.0
Rip-Cre STZ
LATS2fl/fl STZ
β-LATS2-/- STZ
E
Ins/glucose ratio
2.0
1.5
1.0
0.5
0.0
Rip-Cre STZ
LATS2fl/fl STZ
β-LATS2-/- STZ

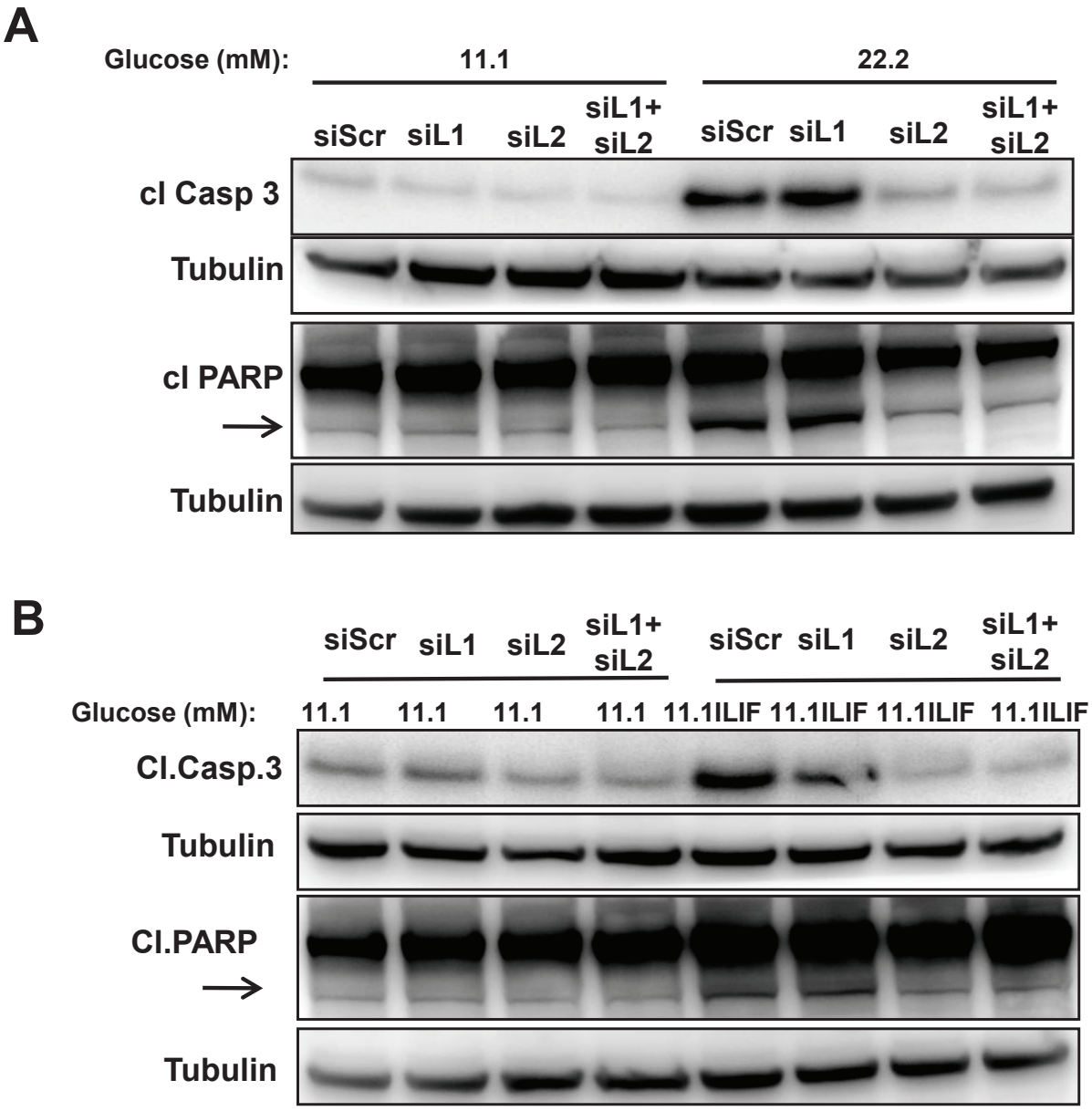
Figure 7



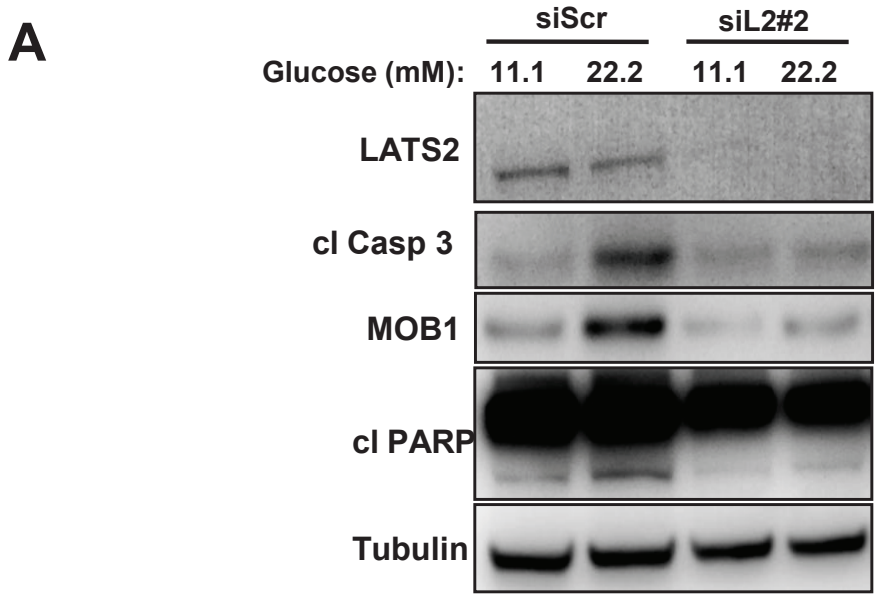
Supplementary Figure 1. LATS2 induces β-cell apoptosis in INS-1E cells. Representative Western blot of INS-1E cells transduced with Ad-LacZ or Ad-LATS2 for 48h.

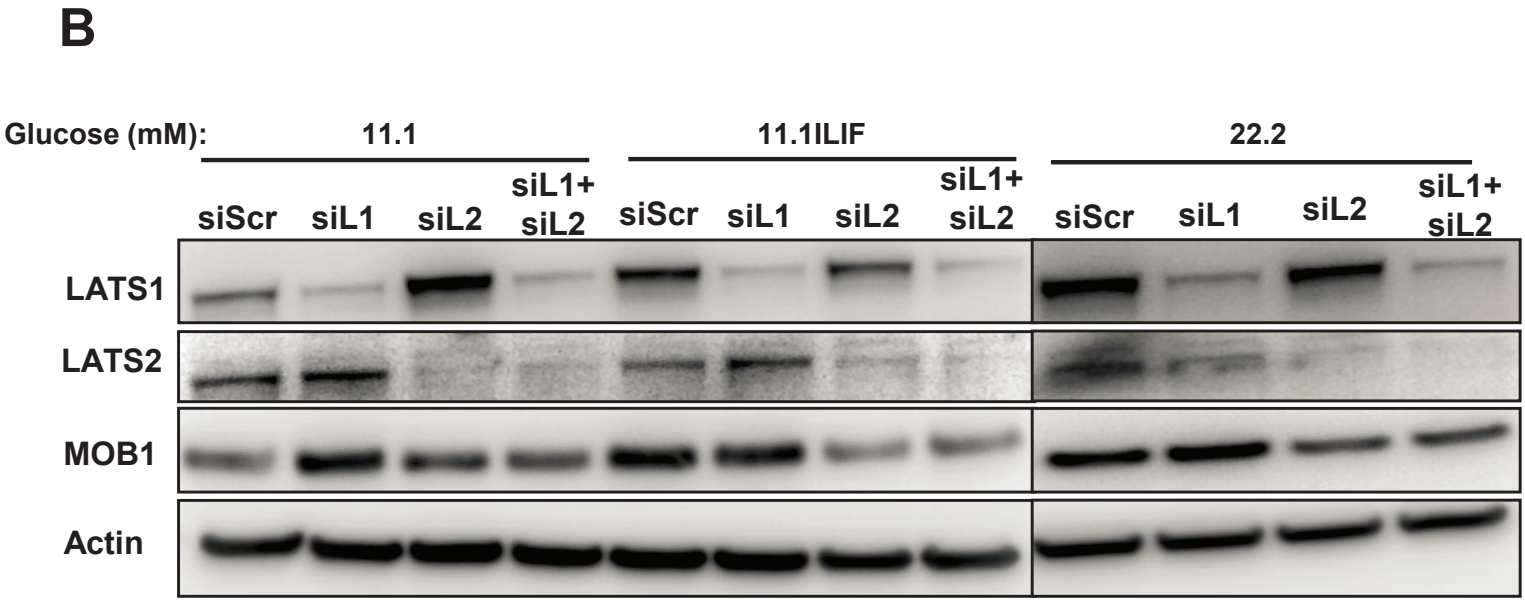


Supplementary Figure 2. Effect of LATS2 on human islets genes. Human islets were transduced with Ad-LacZ or Ad-LATS2 for 48h. RT-PCR analysis of (A) LATS2 and (B) islet genes Insulin, Pdx1, Neurod1, MafA, Glis3, Slc2a2, GCK, Nkx6.1, Nkx2.2, GCG, KCNG11 and Abcc8 normalized to cyclophilin (PPIA). Data are expressed as means ± SEM. (Insulin, Pdx1, Neurod1, MafA, Glis3, Slc2a2, GCK, NKx6.1, Bkx6.2: N=11; GCG: N=4; KCNJ11, ABcc8, N=5)

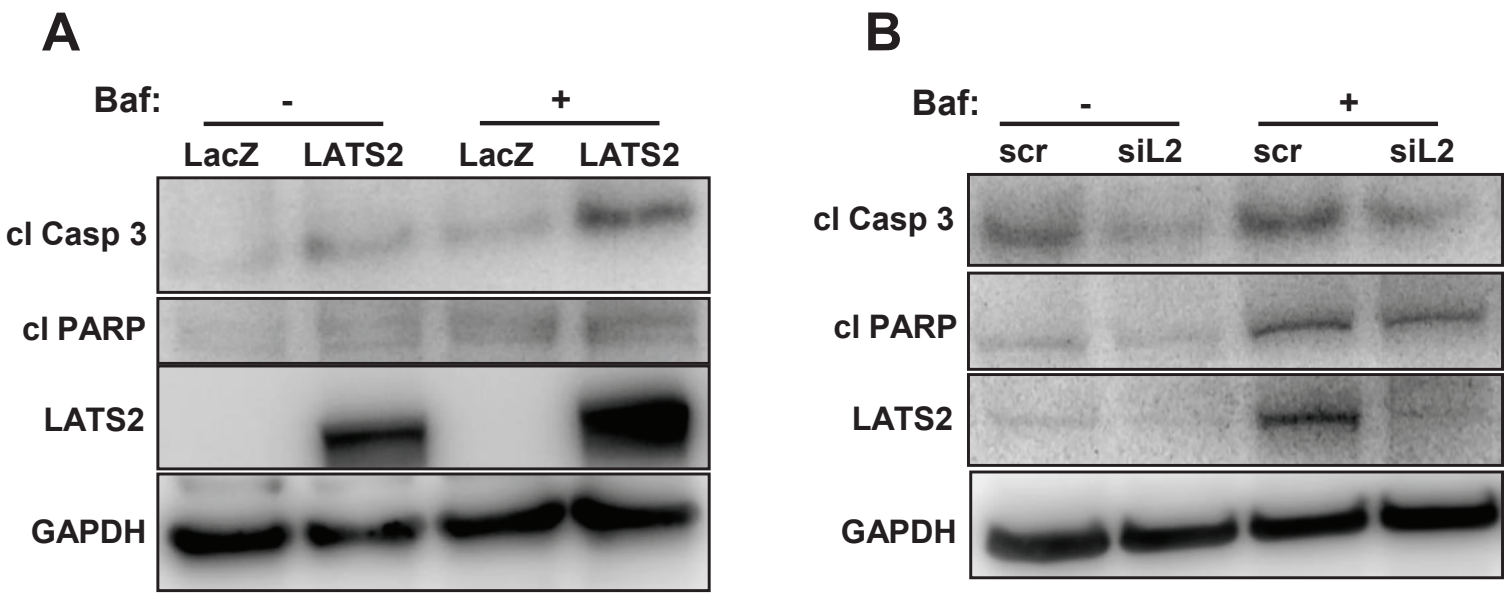


Supplementary Figure 3. LATS2 but not LATS1 knockdown protects from β-cell apoptosis. (A, B) Representative Western blots of INS-1E cells transfected with LATS1 and/or LATS2 siRNA or control siScr and treated with (A) the 22.2 mM glucose, or (B) 2 ng/mL IL1β (IL) plus 1000U/mL IFNγ (IF) for 48 h.

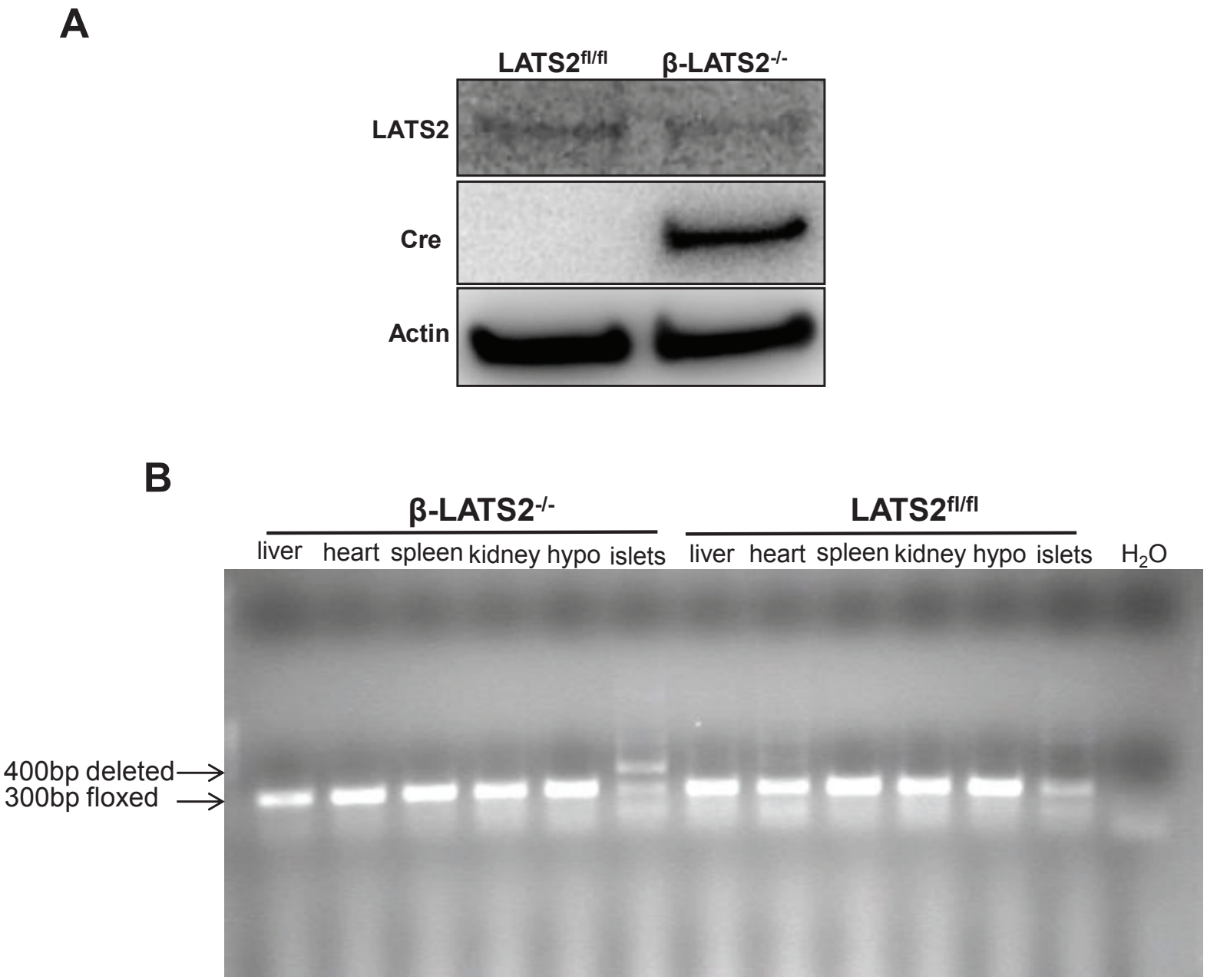




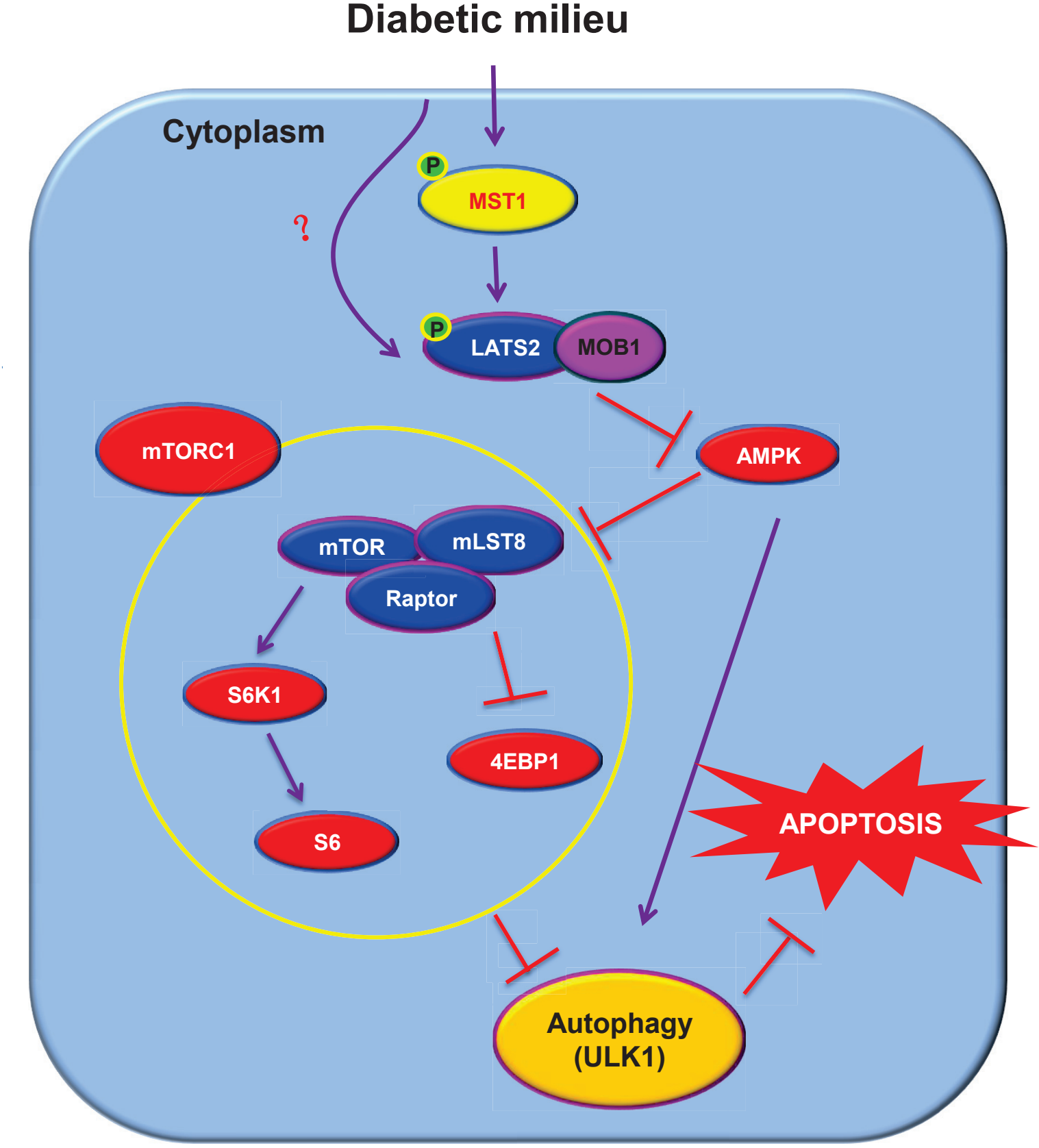
Supplementary Figure 4. LATS2 knockdown protects from β-cell apoptosis. (A) Representative Western blot of INS-1E cells transfected with LATS2 siRNA (second pool #2) or control siScr and treated with the 22.2 mM glucose for 48 h. (B) Representative Western blots of INS-1E cells transfected with LATS1 and/or LATS2 siRNA or control siScr and treated with the 22.2 mM glucose, or 2 ng/mL IL1β (IL) plus 1000U/mL IFNγ (IF) for 48 h.



Supplementary Figure 5. LATS2 regulates defective autophagy-induced β-cell apoptosis in GFP-LC3 expressing INS-1E cell line. (A) Stable GFP-LC3 expressing INS-1E cells were transduced with Ad-LacZ or Ad-LATS2 and treated with 20 nM Bafilomycin A1 (Baf) for 4 h. (B) Stable GFP-LC3 expressing INS-1E cells were tranfected with siLATS2 or siScr control and treated with 20 nM Baf for 4 h.



Supplementary Figure 6. Characterization of β-cell specific LATS2 knockout mice (β-LATS2$^{-/-}$). (A) Representative Western blot analysis of protein lysates from the islets of β-LATS2$^{-/-}$ and LATS2$^{fl/fl}$ control mice (N=3). (B) Genomic PCR analysis of Cre-mediated LATS2 gene deletion in DNA isolated from liver, heart, spleen, kidney, hypothalamus and pancreatic islets of β-LATS2$^{-/-}$ and LATS2$^{fl/fl}$ control mice



Supplementary Figure 7. Our hypothetical view how LATS2 induces β-cell apoptosis. Under diabetogenic conditions, LATS2 is activated (directly by MST1 or through unknown mechanism). Active LATS2 then binds MOB1 and activates mTORC1 signaling pathway by inhibition of AMPK, leading to defective autophagic flux and subsequently induces β-cell apoptosis.

3. Discussion

3.1 Merlin/NF2 impairs pancreatic β-cell survival

NF2, as a tumor suppressor and upstream regulator of the Hippo pathway, controls organ size and development through regulation of cell proliferation and apoptosis in various cell lines [1]. So far, the role of NF2 in the β-cell- whether NF2 is expressed in the β-cell and whether such expression regulates β-cell death–was not known and was investigated in this study. My data showed that NF2 was expressed in β-cell lines as well as in primary human and mouse islets and its depletion rescued β-cells from apoptosis without compromising β-cell function *in vitro*.

NF2 overexpression promotes Wts and Yki phosphorylation in *Drosophila* cells [2, 3], and conversely, NF2-deficient livers show a dramatic decrease in LATS1/2 and YAP phosphorylation [4, 5]. NF2 regulates LATS1/2 phosphorylation and activation by two different mechanisms: 1) direct activation of the MST-Sav complex and subsequent LATS1/2 phosphorylation and activation by MST1/2 [4], 2) direct recruitment of LATS1/2 to the membrane [5]. NF2 functions as scaffold for MST-induced LATS phosphorylation without altering MST1/2 auto-phosphorylation [5]. In support of the first activation model, co-expression of NF2 and Kibra failed to promote LATS2 phosphorylation in the Sav1-deficient ACHN cells, suggesting that Sav1 is a critical mediator of NF2 input into Hippo signaling [4]. In support of the second alternative model, NF2 activates LATS1/2 phosphorylation without activating the intrinsic kinase activity of MST1/2 in mammals [5]. My data showed that loss of NF2 led to reduced LATS1/2 phosphorylation but not MST1/2 phosphorylation in pancreatic β-cells, suggesting that NF2 might directly bind and regulate LATS1/2 phosphorylation, in accordance with the alternative model of LATS activation by NF2. I also demonstrated that inhibition of β-cell apoptosis through NF2 deficiency could be reversed by LATS2 overexpression, showing that NF2 induced β-cell apoptosis through LATS2.

NF2 is a negative regulator of the mTORC1 pathway. Activation of NF2 induces cell apoptosis and blocks tumor initiation through inhibition of mTORC1 signaling [6, 7]. Conversely, loss of NF2 promotes mTORC1 signaling in malignant mesothelioma and is sensitive to rapamycin [6]. Tumor samples isolated from NF2 patients as well as

NF2-deficient mouse embryonic fibroblasts exhibit constitutive elevated mTORC1, proposing a clinically established negative regulation of mTORC1 by NF2 [7]. Interestingly, mTORC1 signaling was increased in both NF2-deficient INS-1E cells and human islets, suggesting the existence of NF2's inhibitory action on mTORC1 in β cells.

In summary, my data showed a direct protective effect of NF2 depletion in pancreatic β-cells by inhibiting LATS2 but not MST1 activity, which could rescue β-cells from apoptosis without compromising β-cell function. Also, mTORC1 activation might be involved in the pro-survival mechanism of NF2 deficiency. The identification of NF2 as the key upstream regulatory and disease-relevant component of the Hippo signaling provides a novel area for potential therapeutic approaches aiming to block β-cell apoptosis in order to restore a functional pancreatic β-cell mass in diabetes.

3.2 YAP-reconstitution promotes human β-cell proliferation and protects from apoptosis

Transcriptional co-activator YES-associated proteins (YAP) is a critical down-stream effector of Hippo signaling which integrates diverse extra- and intra-cellular signals and translates these cues to functional transcriptional programs. At prenatal developmental stage, pancreatic cell proliferation and cell-type specification is regulated by Hippo signaling. Previous studies show that YAP is not expressed in terminally differentiated mature human and mouse β-cells [8, 9]. Expression of YAP is eventually switched off in the mature endocrine but not in exocrine and duct cells [9]; this correlates with the extremely low rate of β-cell proliferation and β-cell quiescence. The transcription factor neurogenin-3 (Ngn-3) is critical for commitment to the endocrine lineage and is generally associated with islet cells becoming mitotically quiescent [10, 11]. The loss of YAP signals right at the onset of pancreatic Ngn3 expression in mouse and human embryos suggests that YAP is repressed following specification of the endocrine lineage which would reduce the ability of adult β-cells to re-enter the cell cycle and thus limits the regenerative potential of adult β-cells [9, 12].

YAP depletion is sufficient to block pancreatic progenitor cell proliferation [13]. Conversely, overexpression of active YAP induces insulin-producing β-cell

proliferation without affecting β-cell differentiation or function in human cadaver islets [12]. Loss of YAP enhances cell apoptosis, whereas YAP overexpression decreased cell apoptosis under lipotoxic condition in INS-1 832/13 cells [14]. In my study, YAP re-expression promoted robust human β-cell proliferation. I have also observed higher resistance of YAP-overexpressing human islets and INS1-E β-cells to apoptosis under multiple diabetic stressors demonstrating that YAP not only induced adult human β-cell proliferation but also protected β-cells from diabetic milieu-induced apoptosis. Thus, YAP activity is important in tissue regeneration and cell proliferation and transient re-expression of YAP activity might be useful for β-cells that normally do not undergo proliferation and for augmenting the regenerative capability of damaged β-cells under conditions of increased β-cell stress and demand *in vivo*.

3.3 The Hippo kinase LATS2 impairs pancreatic β-cell survival and function

My data showed that LATS2 acts as a pro-apoptotic molecule and contributes to the regulation of different signaling pathways leading to β-cell failure under diabetic conditions. Identified the AMPK-mTORC1-autophagy pathway as downstream pathway of LATS2-induced β-cell apoptosis; LATS2 depletion resulted in resistance to apoptosis induced by diabetogenic conditions *in vitro* and improved glycemia and insulin secretion in the MLD-STZ mouse model of β-cell destruction *in vivo*.

LATS2 plays a crucial role in the regulation of apoptosis. Overexpression of LATS2 induces apoptosis in various cells [15-17]. While LATS2 overexpression induces apoptosis, transverse aortic constriction (TAC)-induced cell apoptosis is inhibited by dominant negative LATS2 (DN-LATS2) over-expression in cardiac myocytes [18]. Considering LATS2 as established pro-apoptotic kinase in various cell types, I aimed to identify whether over-expression or down-regulation of LATS2 affects pancreatic β-cell apoptosis. In my study, overexpression of LATS2 alone was enough to promote β-cell apoptosis in INS-1E cells and isolated human islets *in vitro*. Conversely, loss of endogenous LATS2 led to protection from apoptosis in INS-1E cells as well as in primary human islets under diabetic conditions including pro-inflammatory cytokines, gluco- and lipo-toxicity. Proof-of-principle *in vivo* MLD-STZ experiments showed that β-cell-specific LATS2 deletion protected mice from hyperglycemia and development

of diabetes by improving glucose tolerance and insulin secretion. Thus, LATS2 functions as a pro-apoptotic molecule in the presence of multiple diabetic stimuli, and loss of LATS2 protects from apoptosis under diabetic conditions.

mTORC1 plays important roles in β-cell function and survival. Previous studies have demonstrated that mTORC1 activity is up-regulated under diabetogenic conditions in INS-1E cells *in vitro* and that mTORC1 is involved in palmitate- and glucose-induced β-cell apoptosis [19]. In line with this data, my findings show that mTORC1 is activated in both INS-1E cells and isolated human islets upon prolonged culture with increased glucose concentrations. Several studies have suggested that sustained mTORC1 hyper-activation by nutrient overload promotes insulin resistance [20, 21]. In addition, β-cell apoptosis induced by high glucose or growth factors is associated with activation of mTORC1 in β-cells [22]. It seems that mTORC1 is involved in both insulin resistance and β-cell failure under gluco-lipotoxic conditions by promoting ER stress and other unknown detrimental mechanisms. Also, mTORC1 activity is elevated in pancreatic islets of high fat diet fed and *db/db* mice, which was confirmed by us in an independent study [20, 23]. Here, we also show that mTORC1 activity was increased in pancreatic islets from patients with type 2 diabetes, which is consistent with observations from animal models.

My results showed that inhibition of mTORC1 by an S6K1 inhibitor or knockdown of the mTORC1 critical subunit, Raptor, improved insulin secretion and restored AKT phosphorylation in isolated type 2 diabetic islets as well as in HFD mouse islets. Therefore, mTORC1 activation in pancreatic islets might explain the reduced β-cell mass and function associated with type 2 diabetes. In line with the detrimental effect of sustained activation of mTORC1 under diabetic conditions, my mechanistic data proposed that LATS2 is an important regulator of mTORC1 activity in β-cells. I showed that mTORC1 activity is up-regulated by LATS2 overexpression in β-cells and in primary human islets recapitulating aberrant mTORC1 activation in islets isolated from T2D patients as well as from obese and high-fat diet mice and human islets under T2D-asscociated diabetic conditions. To establish a causative correlation between LATS2 and mTORC1, I overexpressed LATS2 in INS-1E cells and isolated human islets and then treated with rapamycin or a specific S6K1 inhibitor as well as raptor silencing to block mTORC1 signaling. My data showed that LATS2-induced

β-cell apoptosis is suppressed by mTORC1-S6K1 signaling blockade, indicating that mTORC1 mediates LATS2-induced caspase-3 activation and apoptosis in β-cells. LATS2 overexpression reduced insulin secretion in human islets. But whether LATS2-reduced insulin secretion is mediated by mTORC1 signaling we do not know yet.

How does LATS2 promote mTORC1 activation? As a key sensor of cellular energy status, AMPK is activated by nutrient-depleted conditions. AMPK expression and activity is decreased in the STZ-induced T1D mouse model [24]. Recent studies suggest apotential crosstalk between the Hippo pathway and AMPK [25-27]. AMPK can inhibit YAP activity through direct phosphorylation and stabilization of AMOTL1 under energy stress, controlling cellular proliferation and survival [25]. Moreover, AMPK is able to inhibit YAP directly by YAP phosphorylation at Ser94 and indirectly via activation of LATS1/2 kinases [26, 27]. These findings show a critical crosstalk between energy and nutrient homeostasis and the Hippo pathway. Notably, AMPK is a powerful negative regulator of mTORC1. Activated AMPK is able to inhibit mTORC1 signaling through two different mechanisms: a) phosphorylation and activation of tuberous sclerosis complex 2 (TSC2) [28], a negative regulator of mTORC1 and b) inhibitory phosphorylation of Raptor at Ser792 [29]. I hypothesized that LATS2 might activate mTORC1 through inactivation of AMPK. My data showed that LATS2 negatively regulates starvation-induced AMPK activity and its down-stream signaling. Conversely, AMPK activation either through overexpression of the constitutively activated form of the AMPK-alpha subunit or overexpression of setrin2, an endogenous activator of AMPK [30], negatively regulated LATS2-induced mTORC1 activation and β-cell apoptosis. This suggests the presence of a regulatory axis among LATS2, AMPK and mTORC1 signaling pathways, which regulate β-cell survival under metabolically stressed conditions. However, it remains unclear how LATS2 inhibits AMPK activity in β-cells. This warrants further mechanistic investigations.

How does mTORC1 promote β-cell failure? It has been recently shown that chronic mTORC1 activation leads to impairment of β-cell autophagy and mitochondrial dysfunction, which both contribute to β-cell failure [31]. Gluco- or lipo-toxicity induce β-cell death through impairing the intracellular flux of autophagy, which is mediated by

the activation of mTORC1 [32]. Consistently, rapamycin-mediated mTORC1 inhibition stimulates autophagy, increases autophagic flux and inhibits β-cell death [32]. Autophagy is a ubiquitous catabolic pathway involved in both cell survival and death. Defective autophagy flux has been recently proposed as critical contributor of β-cell failure in T2D [33-36]. Autophagy is mechanistically regulated by AMPK and mTORC1 signaling pathways [37-39]. While AMPK negatively regulates autophagy, mTORC1 activation suppresses this process in mammalian cells. The mammalian ULK1 complex consists of ULK1, ATG13, FIP200 and ATG101, which initiates stress- or starvation-induced autophagy [40]. Upon autophagy induction, the ULK1 kinase complex is activated and has been suggested to initiate an autophagy pathway [41, 42]. Overexpression of kinase-dead mutant of ULK1 or knockdown of ULK1 leads to inhibition of autophagy [43, 44]. In addition, during amino-acid and glucose induced autophagy, ULK1 is directly regulated by AMPK and mTORC1, leading to cell growth and proliferation [37, 45]. In my study, inhibition of autophagy flux by bafilomycin or chloroquine promoted β-cell apoptosis. Notably, while LATS2 overexpression further potentiated bafilomycin- or chloroquine-induced β-cell apoptosis, LATS2 silencing rescued β-cell apoptosis. These results suggest that LATS2 might regulate defective autophagy-induced β-cell death. p62, an autophagy receptor for the degradation of ubiquitinated proteins, is normally increased when cellular autophagic flux is disturbed [46]. In line with our data, p62 was upregulated by autophagy inhibitors. Interestingly, both p62 and autophagy marker LC3B were upregulated by LATS2 overexpression and downregulated by LATS2 silencing in primary human islets. Interestingly, endogenous LATS2 was upregulated by autophagy inhibitors in INS-1E cells as well as in isolated human islets; this suggests LATS2 as a substrate of autophagy in β-cells, but the mechanism of action remains to be found.

3.4 Outlook

Despite the milestones which have been reached in this thesis towards the understanding of the Hippo signaling pathway in pancreatic β-cell physiology and pathology, some outstanding questions still exist which need to be answered in future studies:

a) Having established the anti-apoptotic effect of NF2 deficiency in β-cells and isolated human islets *in vitro*, what would be the impact of NF2 ablation in pancreatic β-cell in terms of proliferation and survival *in vivo*? Generation of β-cell-specific NF2 knockout mice would provide further insights into the physiological role of NF2 in β-cells *in vivo*.

b) Unlike other Hippo components, YAP expression is muted in adult human and mouse endocrine cells, including β-cells. What are the mechanisms of this developmental silencing? Will transient YAP re-expression in adult mice enhance functional β-cell proliferation, regeneration, and survival at basal state or under conditions of increased β-cell stress and demand *in vivo*? What could stimulate such YAP expression in β-cells *in vivo*, and could such strategy used for β-cell therapy?

c) How does LATS2 inactivate AMPK signaling? Is this direct or indirect through a regulation of AMPK-upstream regulatory network?

d) Does β-cell-specific LATS2 deletion protect mice from β-cell failure and development of diabetes in mouse models of T2D *in vivo*? Testing β-cell-specific LATS2 knockout mice under conditions of nutrients overload in the HFD model or crossing them with diabetic leptin-receptor deficient *db/db* mice will answer whether LATS2 targeting represents an actual therapeutic target in the context of T2D.

e) Our findings suggest that LATS2 hyperactivity may be associated with β-cell apoptosis and development of diabetes. There is currently no selective LATS2 inhibitor. Hopefully, specific inhibitors will be discovered in the future and will be tested to see whether chemically blockade of LATS2 kinase could inhibit β-cell apoptosis and restore β-cell mass in both T1D and T2D settings.

3.5 Conclusion

I have systematically investigated the role of Hippo signaling pathway components in pancreatic β-cells under physiological and diabetes-relevant conditions. Indeed, my data highlight the importance of expression and activation of Hippo signaling elements in proliferation, survival and insulin secretion of pancreatic β-cells.

I have specifically shown:

1. A direct protective effect of NF2/Merlin depletion onpancreatic β-cell survival by inhibiting LATS2 but not MST1 phosphorylation in the alternative model of Hippo signaling regulation. The identification of NF2 as the key upstream regulatory and disease-relevant component of the Hippo signaling provides a novel area for potential therapeutic strategiesaiming to block β-cell apoptosis in diabetes.
2. Re-expression of the Hippo terminal effector YAP in adult human β-cells induced proliferation and protected β-cells from apoptosis under a diabetic milieu, suggesting that YAP is a potent factor for both β-cell replication and survival. YAP promoted proliferation and blocked β-cell apoptosis by transcriptional up-regulation of FOXM1 and Trx1/2 proteins.
3. While mTORC2 signalling was diminished, mTORC1 activity was markedly increased in human and mouse type 2 diabetic islets and in islets and β-cells exposed to elevated glucose concentrations, suggesting a reciprocal regulation of different mTOR complexes with functional upregulation of mTORC1 and downregulation of mTORC2. Inhibition of mTORC1-S6K1 signaling improved glucose-induced insulin secretion and restored mTORC2 activity in human and mouse type 2 diabetic islets. This suggests elevated mTORC1 activation as striking pathogenic hallmark of type 2 diabetic islets contributing to impaired β-cell function and survival in the presence of metabolic stress.
4. I have used a multi-model approach to uncover the role of LATS2 on β-cell survival and function. The results show that LATS2 overexpression impaired β-cell survival and function, whereas lack of LATS2 reduced β-cell apoptosis *in vitro* and ameliorated diabetes in the MLD-STZ mouse model. Further biochemical analysis show that LATS2 induced β-cell apoptosis was mediated by AMPK-mTORC1 signaling. Blocking of LATS2 may be a successful strategy to improve β-cell survival and function in diabetes.

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4. Appendix

4.1 MST1 is a key regulator of beta cell apoptosis and dysfunction in diabetes

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Contribution:

Performed the experiment and analyzed data (Figure 6e).

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MST1 is a key regulator of beta cell apoptosis and dysfunction in diabetes

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Apoptotic cell death is a hallmark of the loss of insulin-producing beta cells in all forms of diabetes mellitus. Current treatments fail to halt the decline in functional beta cell mass, and strategies to prevent beta cell apoptosis and dysfunction are urgently needed. Here, we identified mammalian sterile 20–like kinase-1 (MST1) as a critical regulator of apoptotic beta cell death and function. Under diabetogenic conditions, MST1 was strongly activated in beta cells in human and mouse islets and specifically induced the mitochondrial-dependent pathway of apoptosis through upregulation of the BCL-2 homology-3 (BH3)-only protein BIM. MST1 directly phosphorylated the beta cell transcription factor PDX1 at T11, resulting in the latter's ubiquitination and degradation and thus in impaired insulin secretion. MST1 deficiency completely restored normoglycemia, beta cell function and survival *in vitro* and *in vivo*. We show MST1 as a proapoptotic kinase and key mediator of apoptotic signaling and beta cell dysfunction and suggest that it may serve as target for the development of new therapies for diabetes.

Pancreatic beta cell death is the fundamental cause of type 1 diabetes (T1D) and a contributing factor to the reduced beta cell mass in type 2 diabetes (T2D)[1–4]. In both cases, the mechanisms of beta cell death are complex and as yet not fully defined. Thus, multiple triggering factors have been identified; these factors initiate a variety of signaling cascades that affect the expression of apoptotic genes, leading to subsequent beta cell failure. In T1D, autoimmune destruction of insulin-producing beta cells and critically diminished beta cell mass are hallmarks of the disease[2]. Beta cell destruction occurs through immune-mediated processes; mononuclear cell infiltration in the pancreatic islets and interaction between antigen-presenting cells and T cells lead to high local concentrations of inflammatory cytokines, chemokines, reactive oxygen species and other apoptotic triggers (for example, the perforin and Fas–Fas ligand systems)[2]. In T2D, beta cell dysfunction and reduced beta cell mass are the ultimate events leading to the development of clinically overt disease in insulin-resistant individuals. Beta cell destruction is caused by multiple stimuli including glucotoxicity, lipotoxicity, proinflammatory cytokines, endoplasmic reticulum stress and oxidative stress[5]. Unfortunately, although it has been demonstrated that even a small amount of preserved endogenous insulin secretion has great benefits in terms of clinical outcome[6], none of the currently widely used antidiabetic agents target the maintenance of endogenous beta cell mass.

Beta cells are highly sensitive to apoptotic damages induced by multiple stressors such as inflammatory and oxidative assault, owing at least in part to their low expression of cytoprotective enzymes[7]. The initial trigger of beta cell death still remains unclear; it follows an orchestra of events, which makes the initiation of beta cell death complex and its blockade difficult to successfully achieve *in vivo*. Therefore, the identification of a common key regulator of beta cell apoptosis would offer a new therapeutic target for the treatment of diabetes.

The identification of the genes that regulate apoptosis has laid the foundation for the discovery of new drug targets. MST1 (also known as STK4 and KRS2) is a ubiquitously expressed serine-threonine kinase that is part of the Hippo signaling pathway and involved in multiple cellular processes such as morphogenesis, proliferation, stress response and apoptosis[8,9]. MST1 is a target and activator of caspases, serving to amplify the apoptotic signaling pathway[10,11]. Thr183 in subdomain VIII of MST1 has been defined as a primary site for the phosphoactivation and the autophosphorylation of MST1 and is essential for kinase activation. Both phosphorylation and caspase-mediated cleavage are required for full activation of MST1 during apoptosis. MST1 promotes cell death through regulation of multiple downstream targets such as LATS1 and LATS2, histone H2B and members of the FOXO family, as well as through induction of stress kinase c-Jun-N-terminal kinase (JNK) and activation of caspase-3 (refs. 9,12,13).

Genetic mutations and/or metabolic disturbances can alter protein networks and thereby disrupt downstream signaling pathways that are essential for beta cell survival and function. The transcription factor pancreatic duodenal homeobox-1 (PDX1, previously called IPF1, IDX1, STF1 or IUF1)[14,15] is a key mediator of beta cell development and function[16]. In humans, mutations in the *PDX1* gene can predispose individuals to develop maturity onset diabetes of the young, type 4 (MODY 4)[17], suggesting a critical role for PDX1 in

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mature beta cells; reduced PDX1 expression affects insulin production and secretion and predisposes to beta cell apoptosis[16,18].

Because MST1 acts as common mediator in multiple apoptotic signaling pathways, we hypothesized that it is an initiating trigger of apoptotic signaling in beta cells. MST1 depletion completely restored normoglycemia and insulin secretion and prevented diabetes progression. These findings suggest that MST1 could be a fundamental target for diabetes therapy.

RESULTS

MST1 is activated in diabetes

To test whether MST1 activation is correlated with beta cell apoptosis, we exposed isolated human and mouse islets and the rat beta cell line INS-1E to a complex diabetogenic milieu. MST1 activity was highly upregulated in these cells under these conditions (created through incubation with the cytokines interleukin-1β (IL-1β) and interferon-γ (IFN-γ) (IL/IF), upon chronic exposure to increasing glucose concentrations (22.2 and 33.3 mM) or palmitic acid, or upon exposure to acute oxidative stress from hydrogen peroxide) (**Fig. 1a–c** and **Supplementary Fig. 1a**,**b**). The upregulation of MST1 occurred by both caspase-mediated cleavage and autophosphorylation (yielding MST1 phosphorylated on T183 (pMST1)). This was accompanied by higher phosphorylation of histone H2B as well as induction of JNK signaling (**Fig. 1a–c**). In contrast, short-term culture with high glucose concentrations (11.2, 22.2 and 33.3 mM) induced neither apoptosis nor MST1 cleavage and phosphorylation (**Supplementary Fig. 1d**). MST1 was also activated in islets from subjects with T2D (**Fig. 1d**), obese diabetic *Lepr^{db}* mice (*db/db* mice, **Fig. 1e**) and hyperglycemic mice fed with a high-fat, high-sucrose diet (HFD) for 16 weeks (**Supplementary Fig. 1c**). This activation correlated directly with beta cell apoptosis as described previously[19], as whenever MSTI was induced, apoptosis was also higher. To confirm the beta cell–specific upregulation of MST1, we performed double immunostaining for pMST1 and insulin in pancreatic islets from subjects with poorly controlled T2D (**Fig. 1d**) and pancreatic islets from *db/db* mice (**Fig. 1e**) and found pMST1 staining in beta cells, whereas there was almost no signal in cells from subjects without diabetes or control mice.

Caspase-3 and JNK act not only as downstream targets but also as upstream activators of MST1 through cleavage- and phosphorylation-dependent mechanisms[12,20], and they may initiate a vicious cycle and a proapoptotic signaling cascade in beta cells. Using inhibitors of JNK (SP600125) and caspase-3 (z-DEVD-fmk) and siRNA to caspase-3, we found that both JNK and caspase-3 were responsible for stress-induced MST1 cleavage by diabetic stimuli in human islets and INS-1E cells (**Supplementary Fig. 1e–h**), suggesting that MST1 induces a positive feedback loop with caspase-3 under diabetogenic conditions.

Because phosphatidylinositol-3 kinase (PI3K)-AKT signaling is a key regulator of beta cell survival and function[21,22] and MST1 signaling is negatively regulated by this pathway in other cell types[23,24], we hypothesized that AKT is an important negative regulator of MST1. Maintaining AKT activation through either exogenous addition of mitogens such as glucagon-like peptide-1 (GLP1) or insulin or overexpression of constitutively active AKT1 (Myr-AKT1, containing a myristoylation sequence and HA tag) inhibited glucose- and cytokine-induced phosphorylation of MST1, MST1 cleavage and apoptosis (**Fig. 1f** and **Supplementary Fig. 2a–d**). As GLP1 and insulin exert their cell survival actions primarily through the PI3K-AKT pathway[21,25], we tested whether inhibition of this pro-survival signaling might enhance MST1 activation. PI3K and AKT were inhibited by LY294002, and triciribine (an AKT inhibitor) led to lower levels of phosphorylation of Gsk3 and Foxo1, two well-characterized AKT substrates, and induced MST1 activation (**Fig. 1g**,**h** and **Supplementary Fig. 2e**). We further corroborated these findings using siRNA against AKT, which led to a critical upregulation of MST1 activity and potentiated cytokine-induced phosphorylation of MST1 and beta cell death (**Supplementary Fig. 2f**). MST1 overexpression also diminished insulin-induced AKT phosphorylation and, conversely, there was higher AKT phosphorylation in MST1-depleted beta cells (**Fig. 1i**). Knockdown of MST1 expression antagonized the apoptotic effect of AKT inactivation in INS-1E cells, implicating endogenous MST1 in the apoptotic mechanism induced by PI3K-AKT inhibition (**Supplementary Fig. 2g**,**h**). In summary, these results suggest that MST1 is activated in prodiabetic conditions *in vitro* and *in vivo*, antagonized by PI3K-AKT signaling and dependent on the JNK- and caspase-induced apoptotic machinery.

MST1 induces beta cell death

MST1 overexpression alone was also sufficient to induce apoptosis in human and rat beta cells (**Fig. 2a–c**). To investigate pathways that potentially contribute to MST1-induced beta cell apoptosis, we overexpressed MST1 in human islets and rat INS-1E cells through an adenoviral system, which efficiently upregulated MST1, activated JNK and induced beta cell apoptosis, as determined by an increased number of TUNEL-positive beta cells as well as caspase-3 activation and cleavage of poly-(ADP-ribose) polymerase (PARP), a downstream substrate of caspase-3 (**Fig. 2a–c**). Previous data proposed a role for the mitochondrial pathway in MST-dependent signaling[26,27]. Evaluation of established mitochondrial proteins in MST1-overexpressing islets and INS-1E cells showed cleavage of the initiator caspase-9, release of cytochrome *c*, induction of proapoptotic BAX and a decline in antiapoptotic BCL-2 and BCL-xL levels (**Fig. 2b**,**c** and **Supplementary Fig. 3a**), which led to a reduction of BCL-2/BAX and BCL-xL/BAX ratios. Notably, MST1-induced caspase-3 cleavage was reduced by treatment of human islets with the Bax inhibitor peptide V5 (**Fig. 2d**), which has been shown to promote beta cell survival[28]; together, these findings emphasize that MST1-induced apoptosis proceeds via the mitochondrial-dependent pathway. We also analyzed the expression of BH3-only proteins as regulators of the intrinsic cell death pathway[29]. Of these, BIM was robustly induced, whereas other BH3-only protein levels remained unchanged (**Fig. 2b**,**c** and **Supplementary Fig. 3b**). To assess whether kinase activity of MST1 is required for altering mitochondrial-dependent proteins and induction of apoptosis, we overexpressed a kinase-dead mutant of MST1 (K59R; dominant-negative MST1 (ref. 30)) in human islets. Unlike wild-type (WT) MST1, MST1-K59R did not change the levels of BIM, BAX, BCL-2, BCL-xL and caspase-3 cleavage (**Supplementary Fig. 3c**). We next determined whether BIM is a major molecule whose action would override the proapoptotic action of MST1. Indeed, BIM depletion led to a significant reduction of MST1-induced apoptosis in human islets (**Fig. 2e**,**f**).

Overexpression of MST1 further potentiated glucose-induced apoptosis in beta cells in a BIM-dependent manner (**Supplementary Fig. 3d**). BIM is regulated by the JNK[31] and AKT[32] signaling pathways. MST1-induced increase in BIM and subsequent caspase-3 cleavage was prevented by JNK inhibition through overexpression of dn-JNK1 (**Fig. 2g**) or by the JNK inhibitor (**Supplementary Fig. 3e**), which suggests that MST1 uses JNK signaling to mediate BIM upregulation and induction of apoptosis. We confirmed the involvement of AKT in the regulation of MST1-induced apoptosis by overexpressing

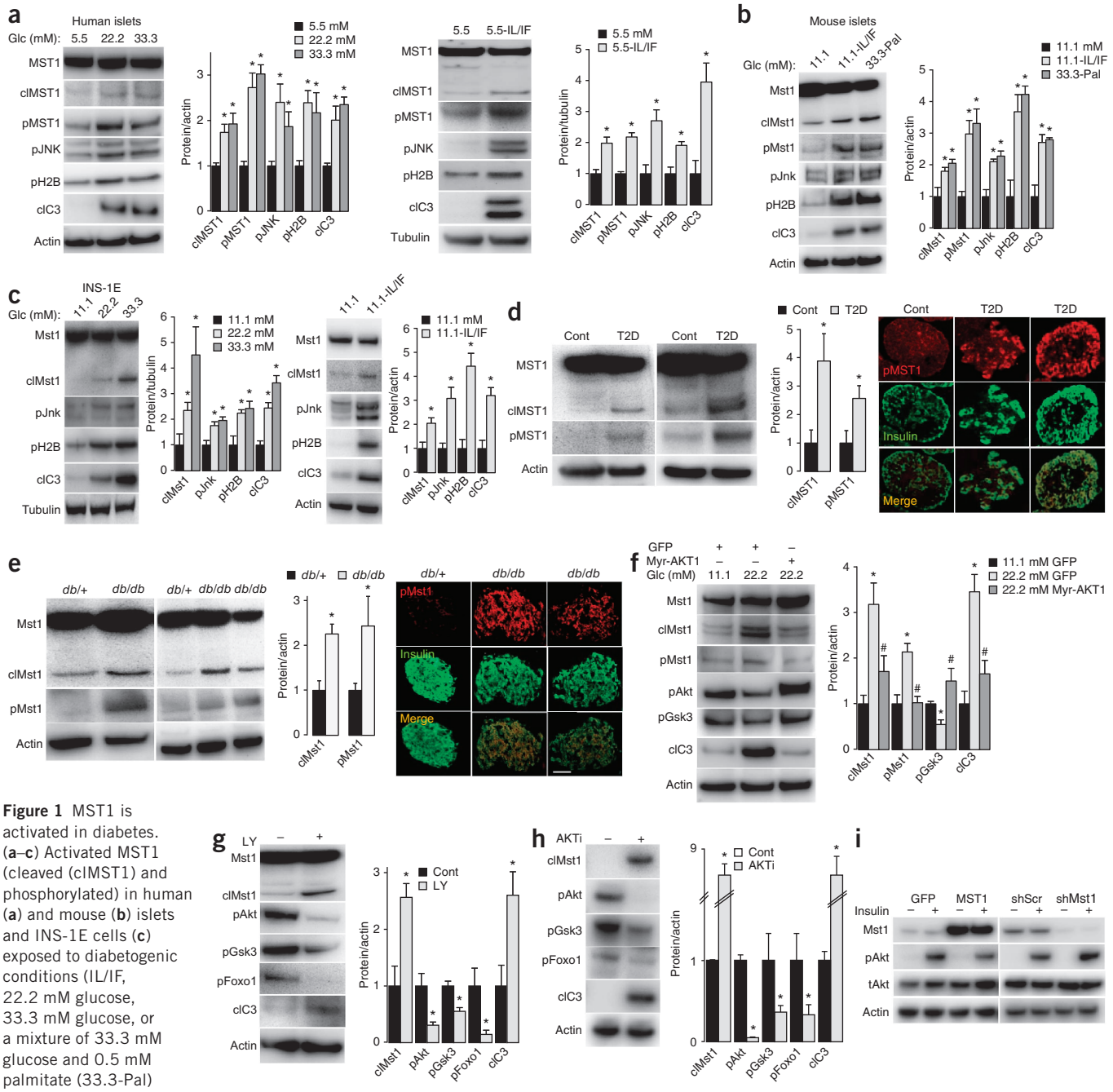

Figure 1 MST1 is activated in diabetes. (**a**–**c**) Activated MST1 (cleaved (clMST1) and phosphorylated) in human (**a**) and mouse (**b**) islets and INS-1E cells (**c**) exposed to diabetogenic conditions (IL/IF, 22.2 mM glucose, 33.3 mM glucose, or a mixture of 33.3 mM glucose and 0.5 mM palmitate (33.3-Pal) for 72 h). Western blots of MST1, pMST1, pJNK, pH2B and caspase-3 cleavage (cIC3) and densitometry analyses are shown. Cont, control; Glc, glucose. (**d**,**e**) Activated MST1 in islets. Human isolated islets from nondiabetic control subjects ($n = 7$) and subjects with T2D ($n = 4$, all with documented fasting plasma glucose >150 mg/dl) (**d**) and from 10-week-old diabetic *db/db* mice ($n = 5$) and their heterozygous littermates (*db/+*, $n = 5$) (**e**). Left, western blots of MST1 and pMST1 and densitometry analyses. Right, double immunostaining for pMST1 (red) and insulin (green) in sections from human isolated islets from nondiabetic control subjects and subjects with T2D and from 6-week-old diabetic *db/db* mice (representative analyses from 10 pancreases from subjects with T2D and >10 pancreases from control subjects and from 7 *db/db* mice and 7 heterozygous controls are shown). Scale bar, 100 μm. (**f**) Western blots of Mst1, clMst1, pMst1, pAkt, pGsk3 and caspase-3 cleavage and densitometry analysis for INS-1E cells transfected with GFP control or Myr-AKT1 expression plasmids exposed to 22.2 mM glucose for 72 h. (**g**) Western blots of Mst1, clMst1, pAkt, pGsk3, pFoxo1 and caspase-3 cleavage and densitometry analysis for INS-1E cells exposed to the PI3K inhibitor LY294002 (LY, 10 μM for 8 h). (**h**) Western blots of clMst1, pAkt, pGsk3, pFoxo1 and caspase-3 cleavage and densitometry analysis for INS-1E cells exposed to the AKT inhibitor triciribine (AKTi, 10 μM for 6 h). (**i**) Western blots of Mst1, pAkt and tAkt for INS-1E cells infected with an adenovirus expressing GFP (Ad-GFP) or MST1 (Ad-MST1) or transfected with shMst1 or shScr control expression plasmids for 48 h, serum-starved for 12 h and then stimulated with insulin. All graphs show densitometry analyses from at least 3 independent experiments normalized to actin or tubulin. All western blots show representative results from at least 3 independent experiments from 3 different donors or mice. Tubulin or actin was used as loading control. Data are expressed as means ± s.e.m. $^{*}P < 0.05$ compared to untreated or nondiabetic control. $^{\#}P < 0.05$ Myr-AKT1 compared to GFP at 22.2 mM glucose.

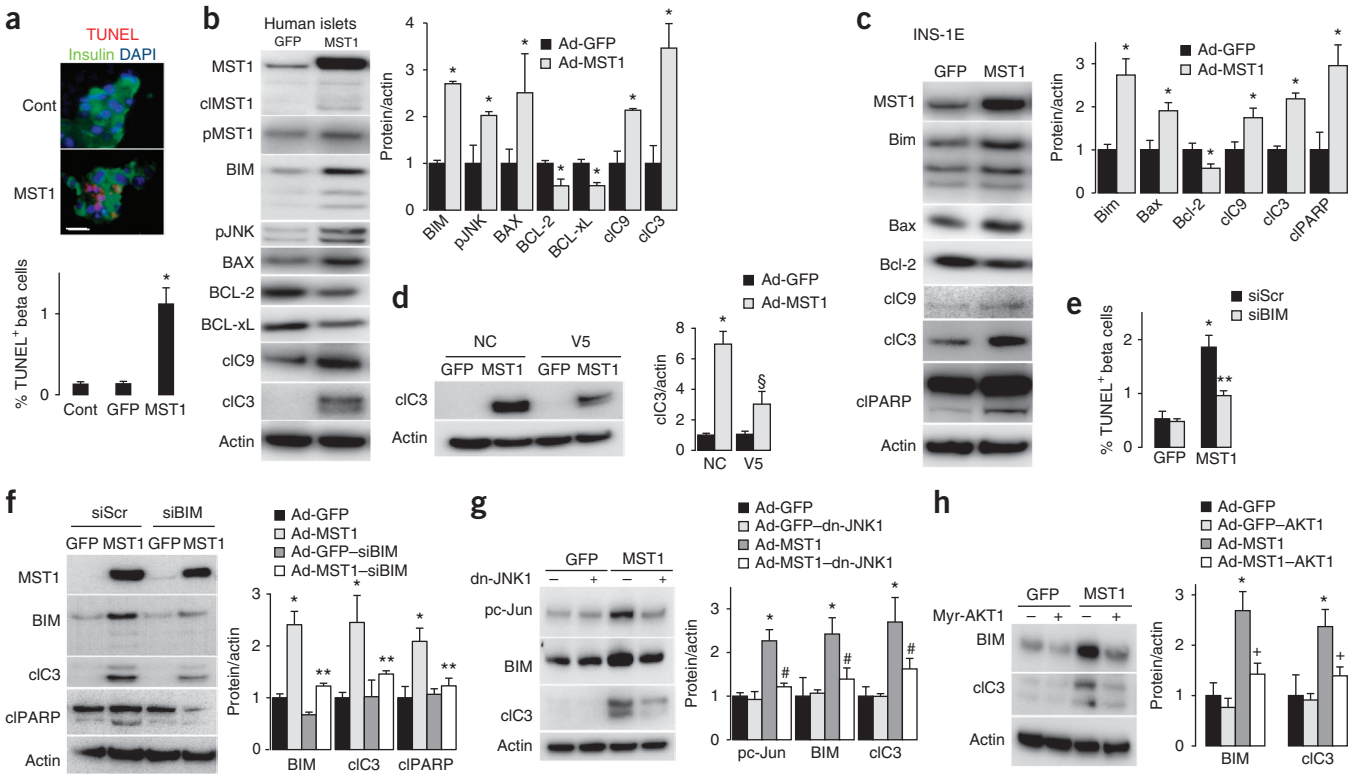

Figure 2 MST1 induces beta cell death. (**a**–**d**) MST1 overexpression in human islets (**a**,**b**) and INS-1E cells (**c**) for 48 h. Triple staining for DAPI (blue), TUNEL (red) and insulin (green) (**a**) TUNEL analysis from an average number of 18,501 insulin-positive beta cells. Scale bar, 100 µm. (**b**,**c**) Adenovirus-mediated upregulation of MST1. Western blots and densitometry analysis of MST1, cleaved MST1, BIM, pJNK, BAX, BCL-2, BCL-xL, cleaved caspase-9 (clC9), cleaved caspase-3 and PARP in human islets (**b**) and INS-1E cells (**c**). (**d**) Western blot and densitometry analysis of caspase-3 cleavage from Ad-GFP– or Ad-MST1–infected human islets exposed to BAX inhibitory peptide V5 or negative control (NC) peptide for 36 h. (**e**,**f**) Human islets transfected with BIM siRNA (siBIM) or control Scr siRNA (siScr) infected with Ad-GFP or Ad-MST1 for 48 h. (**e**) Analysis of cells positive for both TUNEL and insulin out of 10,378 insulin-positive counted beta cells. (**f**) Western blot and densitometry analysis of MST1, BIM and caspase-3 and PARP cleavage. (**g**) Western blot and densitometry analysis of pc-Jun, BIM and caspase-3 cleavage from human islets transfected with GFP or dn-JNK1 expression plasmids and infected with Ad-GFP or Ad-MST1 for 48 h. (**h**) Western blot and densitometry analysis of BIM and caspase-3 cleavage from human islets transfected with GFP or Myr-AKT1 expression plasmids and infected with Ad-GFP or Ad-MST1 for 48 h. All graphs show densitometry analysis from at least 3 independent experiments normalized to actin. All western blots show representative results from at least 3 independent experiments from 3 different donors (human islets). Actin was used as loading control. TUNEL analyses are from 3 independent experiments from 3 different donors. Data are expressed as means ± s.e.m. $^{*}P < 0.05$ MST1 overexpression compared to GFP control, §$P < 0.05$ V5-MST1 compared to MST1, $^{**}P < 0.05$ siBIM-MST1 compared to siScr-MST1, $^{\#}P < 0.05$ dn-JNK–MST1 compared to MST1, $^{+}P < 0.05$ AKT1-MST1 compared to MST1.

both MST1 and Myr-AKT1, which reduced BIM induction and caspase-3 cleavage (**Fig. 2h**), indicating that AKT negatively regulates the downstream target of MST1. These data suggest that MST1 is a critical mediator of beta cell apoptosis through activation of the BIM-dependent intrinsic apoptotic pathway and controlled by AKT and JNK signaling pathways.

MST1 impairs beta cell function by destabilizing PDX1

We hypothesized that MST1 activation may elicit changes in beta cell–specific gene transcription that initiate the process of beta cell failure. Overexpression of MST1 led to a complete loss of glucose-stimulated insulin secretion (GSIS; **Fig. 3a** and **Supplementary Fig. 4a**), which could not be accounted for solely by the induction of apoptosis. Previously, we noted that the critical beta cell transcription factor PDX1, which mediates glucose-induced insulin gene transcription in mature beta cells[16,18], is mislocalized and reduced in diabetes[19]. These changes are subsequently associated with impaired beta cell function and hyperglycemia. Stress-induced kinases such as JNK and glycogen synthase kinase-3 (GSK3) phosphorylate PDX1, antagonizing its activity[33,34], which leads to beta cell failure. Thus, we hypothesized that the drastic reduction in insulin secretion following MST1 overexpression may be mediated by PDX1. PDX1 levels were markedly reduced in response to MST1 overexpression in human islets (**Fig. 3b**) and INS-1E cells (**Supplementary Fig. 4b**). In contrast, MST1 overexpression did not affect the amount of mRNA encoding PDX1 (**Fig. 3b** and **Supplementary Fig. 4b**), suggesting that MST1 may regulate PDX1 at the post-transcriptional level. Analysis of PDX1 target genes demonstrated that overexpression of MST1 significantly downregulated *INS* (*Ins1* or *Ins2* for INS-1E), *SLC2A2* and *GCK* in human islets (**Fig. 3b**) and INS-1E cells (**Supplementary Fig. 4b**). Although SLC2A2 is not the predominant glucose transporter in human beta cells[35], we analyzed its expression to provide comparison to the mouse data.

To gain better insight into the role of MST1 in regulation of insulin secretion, we performed GSIS using two insulin secretagogues: GLP1 and glibenclamide. MST1 overexpression significantly abolished GLP1-enhanced glucose-induced insulin secretion ($P < 0.05$ compared to control condition), whereas glibenclamide-induced insulin secretion was not affected, suggesting that defective insulin secretion may occur at a step upstream of calcium influx (**Supplementary Fig. 4c**).

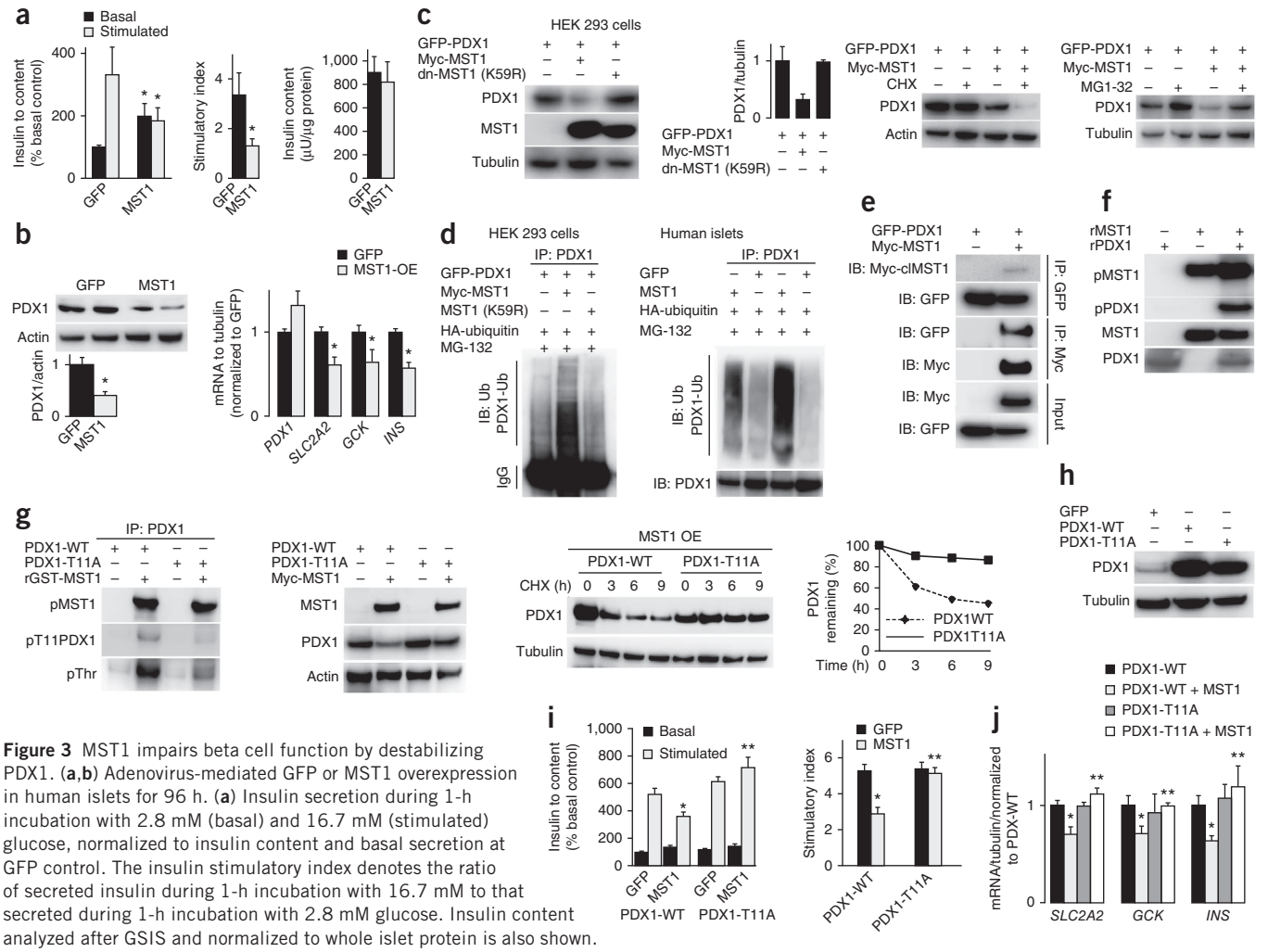

Figure 3 MST1 impairs beta cell function by destabilizing PDX1. (**a**,**b**) Adenovirus-mediated GFP or MST1 overexpression in human islets for 96 h. (**a**) Insulin secretion during 1-h incubation with 2.8 mM (basal) and 16.7 mM (stimulated) glucose, normalized to insulin content and basal secretion at GFP control. The insulin stimulatory index denotes the ratio of secreted insulin during 1-h incubation with 16.7 mM to that secreted during 1-h incubation with 2.8 mM glucose. Insulin content analyzed after GSIS and normalized to whole islet protein is also shown. (**b**) Left, western blot and densitometry analysis of PDX1. Right, RT-PCR analysis of PDX1 target genes *SLC2A2, GCK* and *INS*. (**c**) Western blot and densitometry analysis of PDX1 and MST1 from HEK 293 cells transfected with plasmids encoding Myc-MST1 and GFP-PDX1 with kinase-dead MST1 (dn-MST1, K59R) cotransfected with GFP-PDX1 (left) and western blot of PDX-1 from HEK 293 cells treated with CHX for 8 h at 48 h after transfection, (middle) or treated with the proteasome inhibitor MG-132 for 6 h at 36 h after transfection (right). (**d**) Immunoblotting with ubiquitin-specific antibody after immunoprecipitation with an anti-PDX1 antibody of HEK 293 cells transfected with GFP-PDX1 and hemagglutinin (HA)-ubiquitin (Ub), alone or together with Myc-MST1 or MST1-K59R expression plasmids for 48 h (left) and human islets transfected with HA-ubiquitin and infected with Ad-GFP or Ad-MST1 for 48 h (right; 2 different donors). MG-132 was added during the last 6 h of the experiment. (**e**) Western blot analysis for Myc and GFP with precipitates and input fraction after reciprocal co-immunoprecipitations (using anti-GFP and anti-Myc antibodies) from HEK 293 cells transfected with GFP-PDX1 alone or together with Myc-MST1 for 48 h. (**f**) Western blot of pThr (pan–phosphorylated threonine), MST1 and PDX1 from *in vitro* kinase assay performed with recombinant MST1 (rMST1) and recombinant PDX1 (rPDX1) proteins. (**g**) Left, western blot of pPDX1 (pT11PDX1) and pThr (after immunoprecipitation with anti-PDX1) from HEK 293 cells transfected with PDX1-WT or PDX1-T11A expression plasmids and subjected to an *in vitro* kinase assay using recombinant MST1. Middle, western blot of MST1 and PDX1 from HEK 293 cells transfected with PDX1-WT or PDX1-T11A expression plasmids alone or together with MST1 expression plasmids for 48 h. Right, western blot of PDX1 and densitometry analysis of bands for PDX1-WT or PDX1-T11A cotransfected with MST1 in HEK 293 cells for 36 h and treated with CHX. (**h**) Western blot of PDX1 from human islets transfected with GFP, PDX1-WT or PDX1-T11A expression plasmids. (**i**) Insulin secretion during 1-h incubation with 2.8 mM (basal) and 16.7 mM (stimulated) glucose, normalized to insulin content and basal secretion at control and insulin stimulatory index, which denotes the ratio of secreted insulin during 1-h incubation with 16.7 mM to that secreted at 2.8 mM glucose. (**j**) PDX1 target genes in human islets analyzed by RT-PCR and levels normalized to tubulin and shown as change from PDX1-WT transfected islets from human islets infected with Ad-GFP or Ad-MST1 for 72 h. All western blots show representative results from at least 3 independent experiments from 3 different donors (human islets). Tubulin or actin was used as loading control. RT-PCR (**b**,**j**) and GSIS (**a**,**i**) show pooled results from 3 independent experiments from 3 different donors. Data are expressed as means ± s.e.m. $^{*}P < 0.05$ MST overexpression compared to control, $^{**}P < 0.05$ PDX1-T11A + MST1 compared to PDX1-WT + MST1.

MST1 overexpression had no effect on insulin content (**Fig. 3a** and **Supplementary Fig. 4a**), and thus insulin secretion was normalized on insulin content.

To clarify the mechanism by which MST1 regulates PDX1, we examined the effects of ectopic expression of MST1 and PDX1 in human embryonic kidney (HEK) 293 cells. We found lower PDX1 levels in cells co-overexpressing WT MST1, whereas the kinase-dead MST1-K59R had no effect (**Fig. 3c**). Thus, kinase activity is required for MST1-induced PDX1 degradation. Overexpression of *MST1* also attenuated the transcriptional activity of PDX1 on the rat insulin promoter (RIP), as shown by luciferase assays in PDX1-overexpressing HEK 293 and INS-1E cells (**Supplementary Fig. 4d**).

To discriminate between a transcriptional or translational and a post-translational effect of MST1 on PDX1, we followed the stability of overexpressed PDX1 upon treatment with cycloheximide (CHX), an inhibitor of protein translation. Upon CHX exposure, PDX1 protein levels rapidly decreased when coexpressed with MST1 (**Fig. 3c**), which suggests that MST1 reduced PDX1 protein stability. Consistent with these observations, MST1 overexpression also decreased protein stability of endogenous PDX1 in human islets (**Supplementary Fig. 4e**). In contrast, treatment of PDX1-overexpressing HEK 293 cells with the proteasome inhibitor MG-132 reduced the disappearance of PDX1 (**Fig. 3c**), indicating that MST1 induced activation of the ubiquitin proteasome pathway. Proteasomal degradation of PDX1 has been described before and leads to impaired beta cell function and survival[36].

We next performed *in vivo* ubiquitination assays to determine whether MST1 induces PDX1 ubiquitination. PDX1 cotransfected with MST1, but not with MST1-K59R, was heavily ubiquitinated in HEK 293 cells. We confirmed this in human islets by showing that MST1 overexpression strongly promoted endogenous PDX1 ubiquitination (**Fig. 3d**). Subsequently, we verified a direct interaction between PDX1 and MST1 proteins. Reciprocal co-immunoprecipitations showed the interaction between MST1 and PDX1 in HEK 293 cells cotransfected with GFP-tagged PDX1 and Myc-tagged MST1 (**Fig. 3e**).

We next examined whether a prodiabetic milieu regulates the association between MST1 and PDX1. Notably, both cytokine toxicity and glucotoxicity augment the interaction between MST1 and PDX1 in INS-1E cells (**Supplementary Fig. 4e**). As we observed that PDX1 ubiquitination and degradation required MST1 kinase activity, we tested whether MST1 directly phosphorylates PDX1. *In vitro* kinase assays showed that MST1 efficiently phosphorylated PDX1; these included autoradiography using radiolabeled ^{32}P (**Supplementary Fig. 4f**), as well as nonradioactive kinase assays and western blotting using an antibody specific to pan–phosphorylated threonine (**Fig. 3f**). We confirmed the *in vitro* kinase assays in HEK 293 cells; coexpression of MST1 and PDX1 led to PDX1 phosphorylation (**Supplementary Fig. 4f**). Together, these results establish PDX1 as a substrate for MST1.

We determined the potential MST1-targeted phosphorylation sites on PDX1 theoretically with the NetPhos 2.0 program[37]. This identified six candidate sites within the PDX1 sequence; T11, T126, T152, T155, T214 and T231 (based on the probability that a phosphosite is a substrate of MST1, given as relative score) (**Supplementary Fig. 4g**). These six sites were individually mutated to alanine to generate phosphodeficient constructs as described previously[38]. We subcloned them into pGEX bacterial expression vectors. PDX1-GST fusion proteins with the six different PDX1 mutations were purified from bacteria and used as substrates for MST1 in the kinase assay. With the exception of PDX1-T11A, WT recombinant PDX1 and the other mutants proteins were efficiently phosphorylated at threonine (**Supplementary Fig. 4h**). To confirm this, we transfected all PDX1 mutant plasmids into HEK 293 cells, immunoprecipitated them with a PDX1-specific antibody and incubated them with recombinant MST1 in a kinase assay. MST1 highly phosphorylated PDX1-WT and other mutant proteins, but phosphorylation in the PDX1-T11A mutant was markedly lower (data not shown), indicating that T11 is the major site of phosphorylation by MST1.

In order to confirm T11 as the specific phosphorylation site, we used a phosphospecific antibody against the T11 phosphorylation site in PDX1, which recognized T11 phosphorylation after co-incubation of recombinant PDX1-GST fusion protein with recombinant GST-MST1 (**Supplementary Fig. 4h**). Consistent with this, co-incubation of immunoprecipitated PDX1-WT or PDX1-T11A with recombinant MST1 resulted in robust MST1-induced PDX1-WT phosphorylation at the T11 site (shown by antibody to pT11) and in overall threonine phosphorylation (shown by antibody to pan–phosphorylated threonine); PDX1-T11 phosphorylation was markedly reduced in the PDX1-T11A mutant protein (**Fig. 3g**). We further corroborated this by an *in vivo* kinase assay (**Supplementary Fig. 4h**). Alignment of the amino acid sequences of PDX1 from different species revealed that the T11 site is highly conserved among those species (**Supplementary Fig. 4i**).

If T11 is the specific MST1-induced phosphorylation site of PDX1 and is responsible for beta cell dysfunction, one would expect that mutated PDX1-T11A would reverse beta cell dysfunction. MST1 induced a rapid degradation of exogenous WT PDX1 in the presence of CHX that did not occur in PDX1-T11A mutant–transfected cells (**Fig. 3g**). Furthermore, the half-life of the PDX1-T11A mutant was similar to that of PDX1-WT in the absence of MST1 (data not shown). Consistent with these data, there was less PDX1 ubiquitination in the PDX1-T11A–transfected cells than in those transfected with PDX1-WT (**Supplementary Fig. 4j**).

Because T11 is located within the transactivational domain of PDX1 and to evaluate the functional significance of the T11-dependent ubiquitination and degradation, we assessed transcriptional activity of PDX1. Reduction of PDX1 transcriptional activity occurred only in PDX1-WT– but not in PDX1-T11A–transfected cells (**Supplementary Fig. 4j**). As the T11A mutation of PDX1 prolongs PDX1 stability in the presence of MST1, we asked whether PDX1 stability is directly linked to improved beta cell function. PDX1-T11A mutant overexpression (**Fig. 3h**) normalized MST1-induced impairment in GSIS in human islets (**Fig. 3i**) and INS-1E cells (**Supplementary Fig. 4j**) and restored MST1-induced downregulation of PDX1 target genes (**Fig. 3j** and **Supplementary Fig. 4j**). These findings indicate that MST1-induced PDX1 phosphorylation at T11 leads directly to PDX1 destabilization and impaired beta cell function and suggest that PDX1 is a crucial target of MST1 in the regulation of beta cell function.

MST1 deficiency improves beta cell survival and function

Further analyses aimed to prove whether MST1 not only mediated beta cell death and impaired function *in vitro* but also, when downregulated, allowed for rescue from beta cell failure (**Fig. 4** and **Supplementary Fig. 5**). First, about 80% depletion of MST1 in human islets, achieved with siRNA, protected from cytokine and hydrogen peroxide toxicity as well as glucolipotoxicity; beta cell apoptosis was also inhibited (**Fig. 4a**,**b** and **Supplementary Fig. 5a**). Silencing of *MST1* also significantly reduced BIM upregulation induced by diabetogenic conditions in human islets (**Fig. 4b**,**c** and **Supplementary Fig. 5a**).

Second, beta cell function was greatly improved by *MST1* gene silencing under diabetogenic conditions (**Fig. 4d** and **Supplementary Fig. 5**). Notably, IL/IF- and high glucose + palmitate (HG/Pal)-induced cleavage of caspase-3 and caspase-9 and phosphorylation of H2B was lower in MST1-depleted human islets than in control islets (**Fig. 4b**). *Mst1*$^{-/-}$ islets were largely resistant to IL/IF- and HG/Pal-mediated apoptosis, as determined by TUNEL staining (**Fig. 4e**). In addition to the protective effect of *Mst1* knockout on beta cell survival, *Mst1*$^{-/-}$ islets also showed improved GSIS after long-term culture with IL/IF and HG/Pal (**Fig. 4f** and **Supplementary Fig. 5**). To further support the role of MST1 as a main mediator of apoptosis in beta cells, we generated INS-1E cells stably transfected with vectors carrying *Mst1*-targeting shRNA (sh*Mst1*) or scrambled control shRNA (shScr) and found that Mst1 expression in cells stably expressing sh*Mst1* was about 70% lower than that in cells

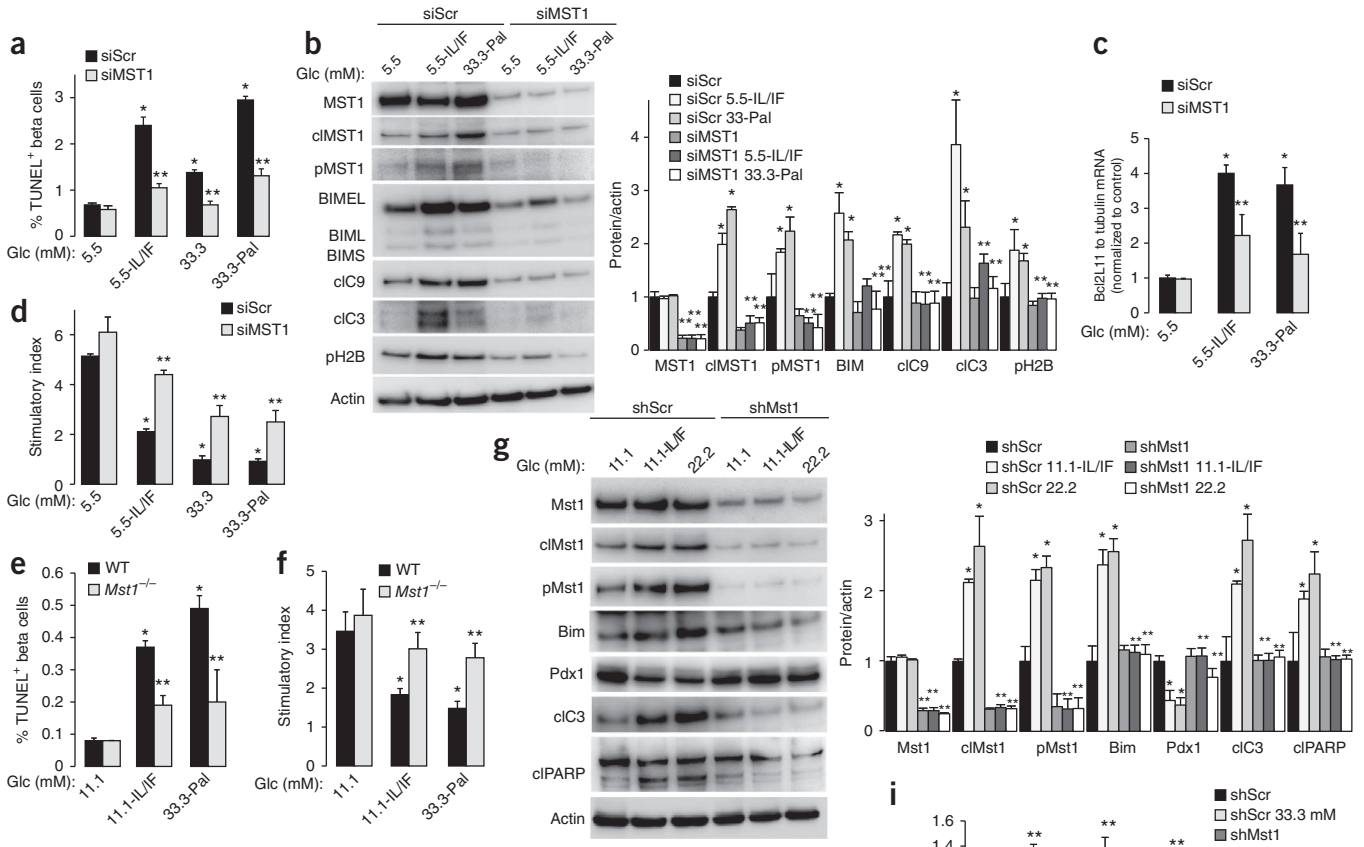

Figure 4 MST1 deficiency improves beta cell survival and function. (**a**–**d**) Analysis of human islets transfected with MST1 siRNA (siMST1) or control siScr and treated with the cytokine mixture IL/IF, 33.3 mM glucose or the mixture of 33.3 mM glucose and 0.5 mM palmitate for 72 h. (**a**) TUNEL-positive beta cells from an average number of 11,390 insulin-positive beta cells for each treatment condition. (**b**) Western blots and densitometry analysis of MST1, pMST1, BIM, pH2B and caspase-9 and caspase-3 cleavage. (**c**) RT-PCR for *BCL2L11* in human islets normalized to tubulin shown as change from siScr control transfected islets. (**d**) Insulin stimulatory index denotes the ratio of secreted insulin during 1-h incubation with 16.7 mM to that secreted during 1-h incubation with 2.8 mM glucose (after the indicated treatments). (**e**) Analysis of TUNEL-positive beta cells from an average number of 24,180 insulin-positive beta cells counted for each treatment condition. (**f**) Insulin stimulatory index denotes the ratio of secreted insulin during 1-h incubation with 16.7 mM to that secreted during 1-h incubation with 2.8 mM glucose from isolated islets from *Mst1*$^{-/-}$ mice and their WT littermates after exposure to the cytokine mixture IL/IF or the mixture of 33.3 mM glucose and 0.5 mM palmitate for 72 h. (**g**–**i**) Western blots and densitometry analysis of Mst1, cIMst1, pMST1, Bim, Pdx1, caspase-3 and PARP cleavage (**g**), insulin stimulatory index (**h**) and RT-PCR analysis of PDX1 target genes *Slc2a2, Gck, Ins1* and *Ins2* normalized to tubulin and shown as change from shScr (**i**) in stable INS-1E clones generated by transfection of vectors for shMst1 and shScr control and treated with the cytokine mixture IL/IF or with 22.2 or 33.3 mM glucose for 72 h. Representative results from 3 independent experiments from 3 different donors (human islets) (**b**,**g**). Actin was used as loading control. TUNEL data (**a**,**e**), GSIS (**d**,**f**,**h**) or RT-PCR (**c**,**i**) show pooled results from 3 independent experiments from 3 different donors (human islets). Data are expressed as means ± s.e.m. $*P < 0.05$ compared to siScr (**a**–**d**), WT (**e**,**f**) or shScr untreated controls (**g**,**h**,**i**); $**P < 0.05$ compared to siScr (**a**–**d**), WT (**e**,**f**) or shScr (**g**–**i**) at the same treatment conditions.

expressing shScr (**Fig. 4g**). We treated INS-1E clones with IL/IF and HG for 72 h. Bim induction, caspase-3 and PARP cleavage in Mst1-depleted cells were significantly lower than that in control cells (**Fig. 4g**). Additionally, *Mst1* silencing also abrogated caspase-3 and PARP cleavage induced by palmitate (**Supplementary Fig. 5b**) and hydrogen peroxide (**Supplementary Fig. 5c**). Cytochrome *c* release was markedly reduced in Mst1-depleted beta cells under diabetogenic conditions (**Supplementary Fig. 5d**,**e**). A second shRNA clone targeting the *Mst1* gene with comparable gene silencing efficiency confirmed the antiapoptotic effect of *Mst1* silencing in INS-1E cells; Mst1 depletion markedly suppressed IL/IF- and HG-induced Bim upregulation and cleavage of caspase-3 and PARP (**Supplementary Fig. 5f**). Confirmation the results of the sh*Mst1* approach, inhibition of endogenous Mst1 activity by overexpression of Mst1-K59R completely inhibited glucose-induced caspase-3 and PARP cleavage in beta cells (**Supplementary Fig. 5g**).

Mst1 deficiency significantly attenuated Pdx1 depletion caused by cytokine or high glucose treatment (**Fig. 4g** and **Supplementary Fig. 5f**), implying that MST1 is indispensable for the reduction in amount of PDX1 induced by a diabetic milieu. Our next objective was to determine whether knockdown of Mst1 expression leads to improvement of GSIS and restoration of Pdx1 target genes in INS-1E cells under diabetogenic conditions. GSIS was significantly improved in Mst1-depleted beta cells (**Fig. 4h** and **Supplementary Fig. 5j**),

whereas levels of Pdx1 target genes, for example, *Slc2a2*, *Gck*, *Ins1* and *Ins2*, were restored in Mst1-depleted INS-1E cells (**Fig. 4i**). These data prove MST1 as determinant for beta cell apoptosis and defective insulin secretion under a diabetic milieu in beta cells *in vitro*.

Mst1 deletion protects from streptozotocin-induced diabetes

As MST1 depletion protected from beta cell apoptosis and restored beta cell function *in vitro*, we hypothesized that Mst1 deficiency may protect against diabetes *in vivo* by promoting beta cell survival and preserving beta cell mass. To test this hypothesis, we used *Mst1*$^{-/-}$ mice. Neither body weight nor food intake differed between *Mst1*$^{-/-}$ mice and their WT (*Mst1*$^{+/+}$) littermates (data not shown). Also, glucose tolerance, insulin tolerance and glucose-induced insulin response did not differ between WT and *Mst1*$^{-/-}$ mice at 2 months of age (**Supplementary Fig. 6a**). However, intraperitoneal (i.p.) glucose tolerance tests (GTTs) and i.p. insulin tolerance tests (ITTs) revealed slight improvement in *Mst1*$^{-/-}$ mice at 6 months of age at 60 min after glucose or insulin injection (**Supplementary Fig. 6b**).

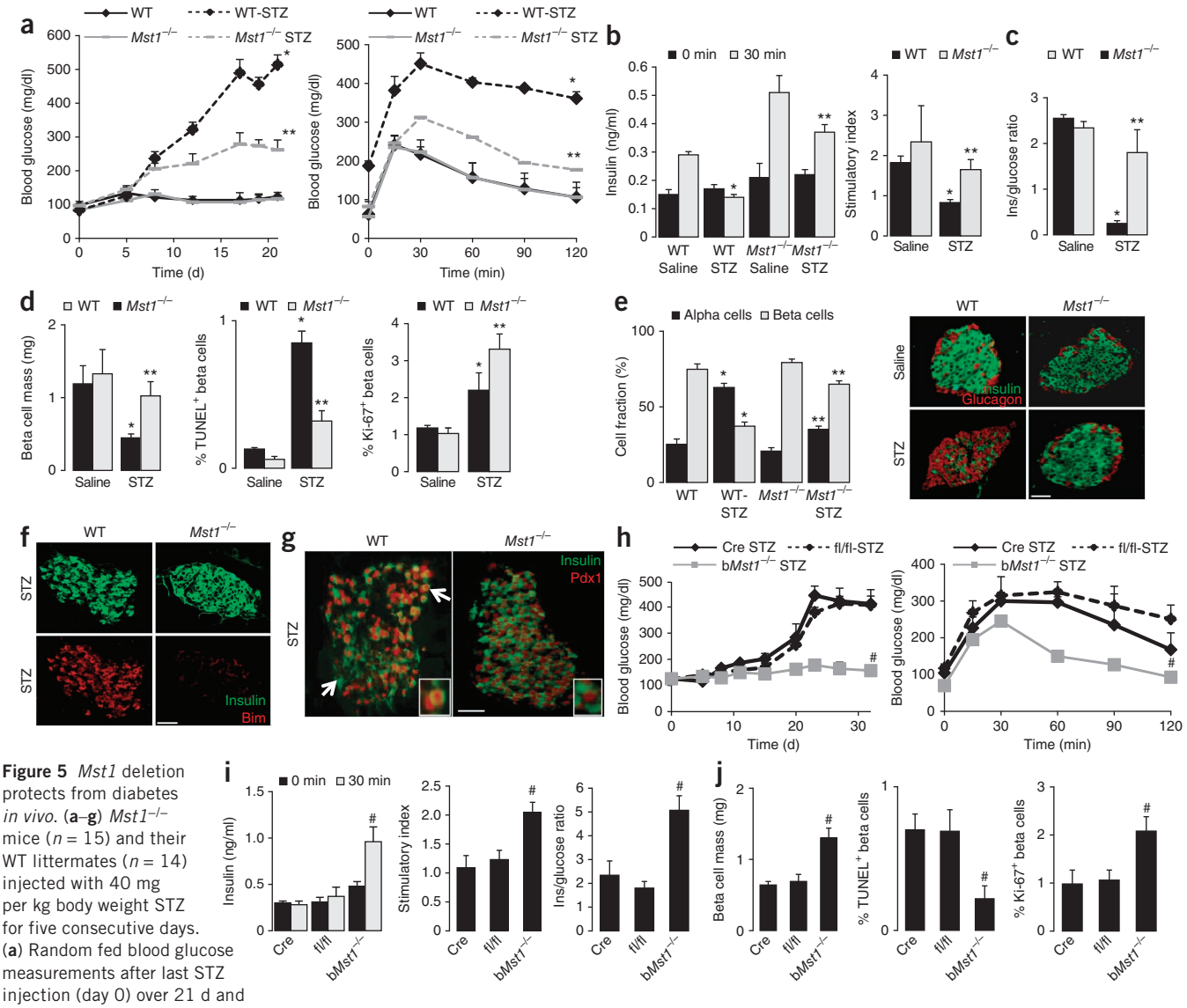

Figure 5 *Mst1* deletion protects from diabetes *in vivo*. (**a**–**g**) *Mst1*$^{-/-}$ mice ($n = 15$) and their WT littermates ($n = 14$) injected with 40 mg per kg body weight STZ for five consecutive days. (**a**) Random fed blood glucose measurements after last STZ injection (day 0) over 21 d and i.p. GTT (ipGTT) performed at day 17. (**b**) Insulin secretion during an i.p. GTT measured before (0 min) and 30 min after glucose injection (left) expressed as ratio of secreted insulin at 30 min to that secreted at 0 min (stimulatory index, right). (**c**) Ratio of secreted insulin and glucose calculated at fed state. (**d**) Beta cell mass and quantitative analyses from triple stainings for TUNEL or Ki-67, insulin and DAPI expressed as percentage of TUNEL- or Ki-67–positive beta cells ± s.e.m. from a mean scored number of 23,121 beta cells for each treatment condition. (**e**) Percentage of alpha cells (stained in red) and beta cells (stained in green) of the whole pancreatic sections from 10 sections spanning the width of the pancreas. Scale bar, 100 µm. (**f**,**g**) Representative double staining for Bim (red, **f**) or Pdx1 (red, **g**) and insulin (green) from MLD-STZ–treated *Mst1*$^{-/-}$ mice and controls killed at day 22. White arrows indicate areas of cytosolic Pdx1 localization and its total absence in WT-STZ–treated mice. Scale bars, 100 µm. (**h**–**j**) b*Mst1*$^{-/-}$ mice with specific deletion in the beta cells using the Cre-*loxP* system ($n = 5$) and RIP-Cre ($n = 3$) and *Mst1*$^{fl/fl}$ controls ($n = 3$) injected with 40 mg per kg body weight STZ for five consecutive days. (**h**) Random fed blood glucose measurements after last STZ injection (day 0) over 32 d and i.p. GTT at day 30. (**i**) Insulin secretion during an i.p. GTT measured before (0 min) and 30 min after glucose injection. Data are expressed as ratio of secreted insulin at 30 min to that secreted at 0 min (stimulatory index). The ratio of secreted insulin and glucose calculated at fed state (right). (**j**) Beta cell mass, TUNEL or Ki-67 analysis, expressed as percentage of TUNEL- or Ki-67–positive beta cells from mice at day 32. Data show means ± s.e.m. $^{*}P < 0.05$ MLD-STZ–treated WT mice compared to saline-injected WT mice, $^{**}P < 0.05$ MLD-STZ–treated *MST1*$^{-/-}$ mice compared to MLD-STZ–treated WT mice. $^{\#}P < 0.05$ MLD-STZ–treated b*Mst*$^{-/-}$ mice compared to MLD-STZ–treated *Mst1*$^{fl/fl}$ or RIP-Cre mice.

We induced diabetes by multiple low-dose (MLD) streptozotocin (STZ) injections in *Mst1*$^{-/-}$ mice and WT controls. Whereas MLD-STZ injection induced progressive hyperglycemia and severely impaired glucose tolerance in WT mice, blood glucose levels were significantly reduced and glucose tolerance was highly improved in *Mst1*$^{-/-}$ mice (**Fig. 5a**). The MLD-STZ treatment led to impaired insulin secretion and a decreased insulin-to-glucose ratio in WT mice, which were significantly restored in *Mst1*$^{-/-}$ mice (**Fig. 5b**,**c**). Islet architecture in MLD-STZ–treated WT mice was disrupted and accompanied by less insulin-positive area, a smaller beta cell fraction, lower islet density, smaller islet size and lower beta cell mass compared to that of non–MLD-STZ–treated mice. In contrast, islet architecture of MLD-STZ–treated *Mst1*$^{-/-}$ mice had a close to normal appearance, and beta cell fraction, islet density and beta cell mass were similar to those in non–MLD-STZ–treated mice (**Fig. 5d**,**e** and **Supplementary Fig. 6c**). Islet size also tended to be higher in MLD-STZ–injected *Mst1*$^{-/-}$ mice than in WT mice, although this effect was not statistically significant (**Supplementary Fig. 6c**).

To elucidate how MST1 deletion may affect beta cell mass, we studied beta cell apoptosis and proliferation. TUNEL staining demonstrated that the rate of apoptosis was dramatically higher in MLD-STZ–treated WT mice than in treated *Mst1*$^{-/-}$ mice; the *Mst1* deletion markedly lowered the rate of apoptosis. Beta cell proliferation was higher in MLD-STZ–treated WT mice compared to untreated WT mice, but treated *Mst1*$^{-/-}$ beta cells showed even higher proliferation, indicative of an improved compensatory capacity (**Fig. 5d** and **Supplementary Fig. 6d**,**e**). No difference in the frequency of proliferating beta cells was observed between islets from *Mst1*$^{-/-}$ mice and those from their WT littermates at basal levels. These results suggest that *Mst1* deletion boosts beta cell mass and islet density predominantly as a result of lower rates of beta cell apoptosis and higher beta cell proliferation in response to diabetogenic stimulation.

To further assess the effect of MLD-STZ, we performed immunohistochemical analyses of insulin and glucagon on pancreatic sections. Islet cells from MLD-STZ–treated WT mice were architecturally distorted, containing significantly fewer insulin-positive cells and proportionally more glucagon-positive cells, which resulted in a higher alpha cell–to–beta cell ratio compared to untreated WT mice (**Fig. 5e**). This is consistent with the previously reported alpha cell hyperplasia in diabetes[39,40]. In contrast, the number of glucagon-positive alpha cells in MLD-STZ–injected *Mst1*$^{-/-}$ islets was not higher and confined to the rim of the islets, suggesting that the architecture of MLD-STZ–injected *Mst1*$^{-/-}$ islets was close to normal (**Fig. 5e**). In line with our *in vitro* results in beta cells, where MST1 acts through changes in BIM, expression of the latter was clearly seen in beta cells in diabetic MLD-STZ–treated WT mice, but not in treated *Mst1*$^{-/-}$ mice (**Fig. 5f**).

We next examined Pdx1 as a beta cell–specific MST1 substrate whose expression is regulated by both its abundance and its subcellular localization in diabetic conditions[19]. Whereas MLD-STZ–treated WT mice showed a reduced nuclear localization of Pdx1 in beta cells, Pdx1 expression was normalized and the prominent nuclear localization important for its functionality reestablished in MLD-STZ–treated *Mst1*$^{-/-}$ mice (**Fig. 5g**). The expression of the Pdx1 target Glut2 in beta cells was largely preserved in the MLD-STZ–treated *Mst1*$^{-/-}$ mice, whereas it was barely detectable in the beta cells of MLD-STZ–treated WT mice (**Supplementary Fig. 6f**). These findings suggest that *Mst1* deletion preserves Pdx1 and Glut2 expression in beta cells and thus preserves the function of beta cells in the MLD-STZ model of diabetes.

To directly assess the protective effect of *Mst1* deletion in MLD-STZ–induced beta cell apoptosis, we treated isolated mouse islets and INS-1E cells with STZ *in vitro* and found that STZ strongly induced phosphorylation of Mst1, Bim expression and ultimately apoptosis, and such apoptotic induction by STZ was attenuated by Mst1 depletion (**Supplementary Fig. 6g**,**h**), consistent with the *in vivo* observations in *Mst1*$^{-/-}$ mice.

To exclude a secondary effect of the *Mst1* deletion on beta cells, we generated mice with beta cell–specific knockout of *Mst1* by the Cre-*loxP* system (hereafter referred to as b*Mst1*$^{-/-}$ mice). These mice contained a null mutation for *Mst1* only in beta cells, as confirmed by western blotting of lysates from isolated islets (**Supplementary Fig. 7a**). b*Mst1*$^{-/-}$ mice were viable, fertile and showed no difference in food intake and body weight (data not shown), glucose tolerance or insulin sensitivity compared to *Mst1*$^{fl/fl}$ mice, which do not express Cre (**Supplementary Fig. 7b**,**c**), or *loxP*-negative mice (RIP-Cre mice; data not shown). To assess whether b*Mst1*$^{-/-}$ mice might also be protected against diabetes, we again used the model of MLD-STZ–induced diabetes. After MLD-STZ treatment, blood glucose levels in *Mst1*$^{fl/fl}$ and RIP-Cre control mice increased gradually (**Fig. 5h**). Whereas both control groups became overtly diabetic, reaching blood glucose levels >400 mg/dl, b*Mst1*$^{-/-}$ mice maintained normal blood glucose levels. *Mst1*$^{fl/fl}$ and RIP-Cre control mice exhibited impaired glucose tolerance; this was notably prevented in b*Mst1*$^{-/-}$ mice (**Fig. 5h**). This protection was accompanied by significant restoration of glucose-induced insulin response and insulin-to-glucose ratio (**Fig. 5i**). Beta cell protection was also confirmed by the considerably higher beta cell mass in the MLD-STZ–treated b*Mst1*$^{-/-}$ mice, resulting from enhanced beta cell survival and proliferation (**Fig. 5j**), compared to *Mst1*$^{fl/fl}$ and RIP-Cre control mice. These data indicate that beta cell–specific disruption of *Mst1* prevented progressive hyperglycemia and improved glucose tolerance in MLD-STZ–treated mice as a result of less apoptosis and restoration of beta cell mass, suggesting that beta cell–specific activation of Mst1 is a key event in the progressive loss of beta cells in diabetes.

Mst1 deletion protects from HFD-induced diabetes

We further confirmed the protective effect of *Mst1* deletion against hyperglycemia and development of diabetes in a mouse model of T2D. We fed b*Mst1*$^{-/-}$ mice and RIP-Cre controls a normal diet (ND) or a HFD for 20 weeks. Mice fed a HFD gained more weight than the ND-fed group. Beta cell–specific disruption of *Mst1* had an effect on neither weight gain nor food intake in both groups (**Supplementary Fig. 7d**,**e**). HFD feeding increased fed and fasted glucose levels (**Fig. 6a**,**b**) and impaired glucose tolerance (**Fig. 6b**) in the HFD-treated RIP-Cre control mice compared to ND-fed mice, whereas HFD-treated b*Mst1*$^{-/-}$ mice showed significantly lower fed and fasted glucose levels as well as improved glucose tolerance (**Fig. 6a**,**b**).

In RIP-Cre mice on a HFD, insulin secretion during i.p. glucose challenge was markedly attenuated compared with that of the ND-fed group. In contrast, HFD-induced impairment in GSIS was dramatically reversed in mice with beta cell–specific deletion of *Mst1* (**Fig. 6c**). For assessing beta cell glucose responsiveness, we isolated islets from all ND- and HFD-fed groups. Whereas GSIS was severely impaired in islets isolated from HFD-treated RIP-Cre mice compared with ND-treated RIP-Cre mice, islets from b*Mst1*$^{-/-}$ mice remained fully responsive to glucose with improved insulin secretion under the HFD (**Fig. 6d**). Consistent with the improved metabolic phenotype of b*Mst1*$^{-/-}$ mice on HFD, b*Mst1*$^{-/-}$ mice had a higher compensatory beta cell mass relative to WT HFD control mice (**Fig. 6e**). The combination of lower rate of beta cell apoptosis and elevated beta cell proliferation (**Fig. 6e**) in b*Mst1*$^{-/-}$ mice on the HFD, which accounts

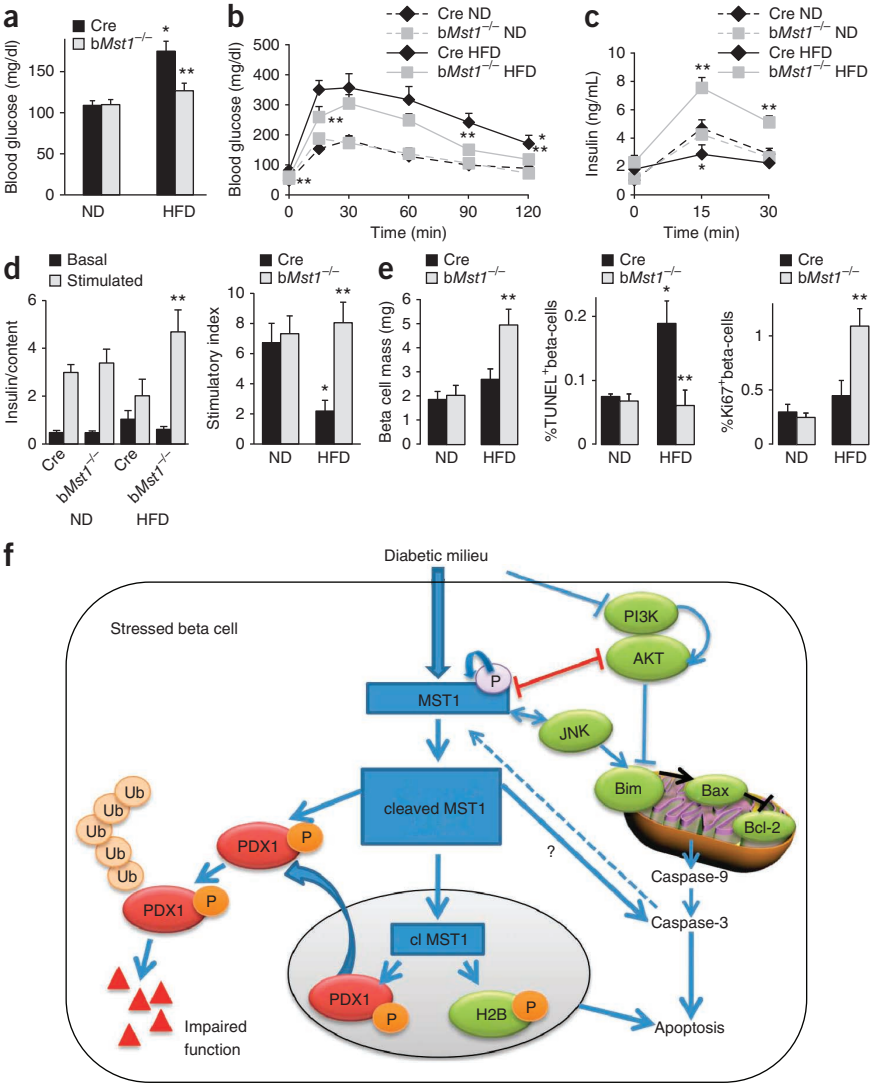

Figure 6 Beta cell–specific disruption of *Mst1* prevents hyperglycemia and HFD-induced diabetes progression. (**a**–**e**) b*Mst1*$^{-/-}$ mice ($n = 12$) and Cre control mice ($n = 12$) fed a ND or a HFD diet for 20 weeks. (**a**–**c**) Random fed blood glucose measurements (**a**), i.p. GTT (**b**) and insulin secretion (**c**) during an i.p. GTT measured before (0 min) and 15 and 30 min after glucose injection. (**d**) Insulin secretion during 1-h incubation with 2.8 mM (basal) and 16.7 mM (stimulated) glucose normalized to insulin content from isolated islets from all 4 treatment groups at week 21, cultured overnight and subjected to an *in vitro* GSIS assay. The insulin stimulatory index denotes the ratio of secreted insulin during 1-h incubation with 16.7 mM to secreted insulin at 2.8 mM glucose. (**e**) Beta cell mass, TUNEL and Ki-67 analysis expressed as percentage of TUNEL- or Ki-67–positive beta cells ± s.e.m. from a mean number of 28,521 scored beta cells for each treatment condition. $^{*}P < 0.05$ HFD-fed Cre mice (Cre HFD) compared to ND-fed Cre mice (Cre ND). $^{**}P < 0.05$ HFD-fed b*Mst1*$^{-/-}$ mice (b*Mst1*$^{-/-}$ HFD) compared to HFD-fed Cre mice (Cre HFD). (**f**) Our view on how diabetic stimuli lead to activation of MST1. Active MST1 triggers cytochrome *c* release and mitochondrial-dependent apoptosis by modulating Bim/Bax/Bcl-2/Bcl-xL through JNK-AKT signaling. Active caspase-9 then triggers cleavage of caspase-3, which triggers the caspase-3–dependent cleavage of MST1 to its constitutively active fragment, which leads to further MST1 activation and processing of caspase-3 by a positive feedback mechanism, and acceleration of beta cell death occurs. Cleaved MST1 translocates to the nucleus and directly phosphorylates PDX1 (we do not exclude the possibility that MST1 targets PDX1 also in cytoplasm) and histone H2B. PDX1 then shuttles to cytosol, where it undergoes ubiquitination and subsequent degradation by proteasome machinery, and beta cell function is impaired. Histone H2B phosphorylation by MST1 also induces chromatin condensation, one of the characteristic features of apoptosis.

for the higher beta cell mass in b*MST1*$^{-/-}$ mice under the HFD, correlates with the improved glucose tolerance and insulin secretion. This is also supported by results from an i.p. ITT: glucose levels were normalized to the basal levels before insulin injection, and b*Mst1*$^{-/-}$ mice and RIP-Cre control mice on HFD showed a similar impairment in insulin sensitivity (**Supplementary Fig. 7f,g**).

To test whether knockdown of MST1 expression could directly rescue beta cells, we transfected islets isolated from 10-week-old obese *db/db* mice and their heterozygous littermates with scrambled control siRNA or *Mst1* siRNA. Whereas Scr siRNA–treated isolated islets from *db/db* mice showed low Pdx1 expression and high caspase-3 cleavage and Bim expression, *Mst1* silencing restored Pdx1, inhibited Bim upregulation and dramatically reduced caspase-3 cleavage (**Supplementary Fig. 8**).

DISCUSSION

Our work shows that MST1 acts as an essential apoptotic molecule in the presence of diabetic stimuli and is a common component in the diverse signaling pathways leading to impaired beta cell survival in diabetes. We identified PDX1 as a beta cell–specific substrate for MST1. PDX1 ubiquitination and subsequent degradation, resulting from inhibitory T11 phosphorylation, is crucial for beta cell dysfunction after MST1 hyperactivation in diabetes (**Fig. 6f**). Deletion of *Mst1* preserves beta cell function and survival providing protection from diabetic insults.

MST1 has a central role in the initiation of cell death[9,41–44]. In line with our data, *Mst1* ablation *in vivo* resulted in resistance to apoptosis induced by tumor necrosis factor-α[45], Fas ligand[46] or IFN-γ[47]. Notably, suppression of endogenous Mst1 by cardiac-specific overexpression of the dominant-negative form of MST1 prevented cardiomyocyte death induced by ischemia-reperfusion[48], which supports the pathophysiological significance of MST1. The diversity of diabetic stimuli by which MST1 is activated in beta cells suggests that this enzyme may be a common component in the many signaling pathways leading to beta cell apoptosis. Although the endogenous molecules that trigger MST1 activation remain unknown, we show that MST1 is highly active in a diabetic environment and induces the mitochondrial-dependent apoptosis pathway in beta cells through targeting BIM, which leads to alterations in BCL-2/BAX or BCL-xL/BAX ratios, cytochrome *c* release, subsequent caspase-9 and caspase-3 cleavage and cell death.

The PI3K-AKT pathway has a critical role in the regulation of beta cell survival. AKT-mediated phosphorylation of multiple substrates positively regulates insulin transcription, insulin secretion and beta cell growth and survival[21,22,49]. Recent studies suggest potential crosstalk between MST1 and AKT[23,24]. MST1 activity is negatively regulated by AKT-mediated phosphorylation at its T120 and T387 residues, which results in inhibition of its cleavage, autophosphorylation, kinase activity and nuclear translocation[23]. On the other hand, MST1 and its cleaved form interact with AKT1 and act as direct AKT1 inhibitors[24]. Our data demonstrate that activation of the PI3K-AKT pathway in beta cells abrogates glucose- and cytokine-induced MST1 activation and beta cell apoptosis, whereas suppression of PI3K-AKT signaling induces MST1 activity and beta cell apoptosis. AKT and MST1 are components of two parallel stress-triggered signaling pathways that functionally antagonize each other. Activated AKT itself downregulates MST1 function in beta cells, indicating the existence of a potential bidirectional crosstalk between these two pathways. Here, we show that MST1 and AKT negatively regulate each other and constitute a stress-sensitive survival pathway. Under acute stress conditions, AKT promoted cell survival by inhibiting MST1, but prolonged stress decreased AKT activity, which allowed for proapoptotic MST1 signaling.

MST1 may affect signal pathways of diabetic stimuli through modulation of transcription factors and gene expression profiles that initiate the process of beta cell failure. In this study, we show that MST1 can physically interact with and phosphorylate PDX1. Targeted disruption of PDX1 in beta cells leads to diabetes, and reducing its expression affects insulin expression and secretion[50]. We have identified the T11 residue of PDX1 as the phosphorylation site used by MST1. Such a kinase-dependent function would be consistent with the comparably low level of PDX1 and high levels of active MST1 in stressed beta cells and pancreases of diabetic mice in our study. PDX1 is restored by deletion of *Mst1* under diabetic conditions. T11 is found in the highly conserved region of PDX1 at the transcription activation domain. T11 phosphorylation of PDX1 by MST1 marks PDX1 for degradation by the proteasome machinery, which would prohibit it from functioning as a transcription factor in the nucleus. In that regard, overexpression of MST1 caused reduction of PDX1 target gene expression and impairment of beta cell function, as assessed by GSIS, whereas mutation of the T11 site allowed PDX1 to be more stabilized and resistant to MST1-induced degradation, restoring PDX1-induced gene expression and improvement of beta cell function. The same site (T11) was previously shown to be targeted by DNA-dependent protein kinase. Consistent with our data, phosphorylation of PDX1 by this kinase results in enhanced PDX1 protein degradation[51]. PDX1 is degraded by the ubiquitin-proteasome pathway; PDX1 C terminal–inhibiting factor-1 (PCIF1) targets PDX1 for ubiquitination and proteasomal degradation by the E3 ubiquitin ligase cullin 3. Ubiquitination of PDX1 regulates its activity, as Pcif1 deficiency normalizes Pdx1 protein levels and improves glucose homeostasis and beta cell function in *Pdx1*$^{+/-}$ mice[36]. Notably, accumulation of polyubiquinated proteins was higher in beta cells of individuals with T2D than in nondiabetic controls[52], highlighting that higher expression of polyubiquinated proteins may contribute to beta cell dysfunction under diabetic conditions.

In mammals, the absolute number of beta cells reflects a dynamic balance between beta cell growth and death. An inadequate expansion of beta cell mass to compensate for the increased insulin demand, followed by the eventual loss of beta cells due to apoptosis, is a hallmark of diabetes[4,53]. This is most apparent in T1D when ongoing autoimmunity causes destruction and consequent loss of beta cells. Through deletion of the MST1-mediated death signal, we have uncovered a deleterious action of MST1 that induces apoptosis in response to diabetic injuries in the immune-mediated beta cell destruction in the MLD-STZ model, a model of beta cell demise that occurs in the absence of insulin resistance. *Mst1* deletion not only prevents MLD-STZ–induced beta cell death but also improves the capacity of beta cells to produce insulin. *Mst1* deletion preserves beta cell mass, improves beta cell function and prevents islet deterioration, as shown by the maintenance of the islet structure, density, size and mass. The observed ability to preserve islet appearance is associated with a protective role for Mst1 deficiency in MLD-STZ–induced beta cell death and in enhancing beta cell proliferation.

Preservation of PDX1 is one mechanism involved in the protection of beta cells by MST1 depletion. This conclusion is strongly supported by our *in vitro* and *in vivo* data; Pdx1 target gene expression is normalized and Glut2 localization is preserved in *Mst1*$^{-/-}$ mice. As both Pdx1 and Glut2 are involved in glucose sensing and GSIS, *Mst1*$^{-/-}$ mice show normal blood glucose levels and high circulating insulin concentrations. STZ enters beta cells via Glut2. It is unlikely that the resistance of *Mst1*$^{-/-}$ mice to MLD-STZ–induced beta cell damage is due to changes in membrane Glut2 expression in beta cells because *Mst1* deletion did not reduce membrane expression of Glut2 in beta cells in mice without STZ treatments.

The high rate of apoptosis in the Mst1-deficient thymocytes further illustrates the cell type–specific variation in the outputs of MST1 signaling. Whereas in thymocytes and T cells deletion of *Mst1* increases the apoptosis rate[54–56], possibly through high levels of reactive oxygen species, Mst1-deficient hepatocytes and microglia exhibit a marked resistance to stress-induced apoptosis[46,47]. Thus, the consequences of Mst1 deficiency need to be established in each cell type and tissue. We used a tissue-specific gene-targeting approach in the current study to provide insights into the biological role of Mst1 in beta cells *in vivo*. It is known that activation and migration of both T cells and macrophages play an important part in islet destruction leading to hyperglycemia in the MLD-STZ model[57]. Infiltrating macrophages and T cells are a major source of the proinflammatory cytokines that promote islet destruction. Thus, the depletion of peripheral T cells in *Mst1*$^{-/-}$ mice[54,55] might be a reason for their protection from MLD-STZ–induced hyperglycemia. We cannot exclude such T cell depletion in our model, but if it occurs, it only has a minor role, as beta cell–specific deletion of *Mst1* in mice completely protected them from hyperglycemia and islet destruction. This shows that *Mst1* ablation in beta cells, but not in other tissues, is a major reason for the protection from MLD-STZ–induced diabetes. Notably, beta cell–specific deletion of *Mst1* in this model led to protection against beta cell apoptosis and diabetes, further underlining the critical role of MST1 in beta cell survival.

The detrimental effects of a long-term HFD on beta cell function and insulin sensitivity leading to glucose intolerance and T2D in mice have been clearly established[58]. As expected, long-term HFD feeding was associated with insulin resistance, glucose intolerance, beta cell dysfunction and loss of compensatory beta cell adaptation. As observed in the MLD-STZ model, in the HFD diabetes model, beta cell–specific *Mst1* deletion results in improved glucose tolerance, insulin secretion and beta cell mass as a result of improved beta cell survival and proliferation, whereas insulin sensitivity is not affected. We have not investigated whether Mst1 is also activated in other organs during diabetes progression, but activated Mst1 has been found in the kidneys of hyperglycemic insulin receptor substrate-2 knockout mice[59] and in epididymal fat pads of HFD-treated mice[60].

Our findings raise the possibility that MST1 hyperactivity is associated with beta cell failure and development of diabetes. Current therapies for the treatment of diabetes mellitus are directed toward alleviating the symptoms of the disease, but there is an urgent medical need for therapies that slow or prevent the loss (rapid in T1D, progressive in T2D) of functional pancreatic beta cell mass. In light of the critical role of MST1 in beta cell failure and initiation of pro-diabetic milieu–induced apoptotic signaling, therapeutic strategies designed to inhibit MST1 activity may both protect beta cells against the effects of autoimmune attack in T1D and preserve beta cell mass and function in T2D.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.A. conceived of the project, designed all and performed most of the experiments, analyzed the data and wrote the paper. F. Paroni provided experimental and technical support and analyzed data. Z.A., S.K., V.K. and T.Y. performed experiments and analyzed data. T.F. provided mutated PDX1 plasmids, W.T. provided *Mst1*$^{-/-}$ and *Mst1*$^{fl/fl}$ mice and J.K.C., F. Pattou and J.O. isolated human islets. K.M. supervised the project and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell culture, treatment and islet isolation. Human islets were isolated from twenty pancreases of healthy organ donors and from five with T2D at the University of Illinois at Chicago or Lille University and cultured on extracellular matrix (ECM)-coated dishes (Novamed, Jerusalem, Israel) as described previously[61]. Informed consent was obtained from all subjects. Islet purity was greater than 95% as judged by dithizone staining (if this degree of purity was not achieved by routine isolation, islets were handpicked). Ethical approval for the use of human islets had been granted by the Ethics Committee of the University of Bremen. Islets from *Mst1*$^{-/-}$ mice and their WT littermates were isolated as described previously[61]. Pancreases were perifused with a Liberase TM (#05401119001, Roche, Mannheim, Germany) solution according to the manufacturer's instructions and digested at 37 °C, followed by washing and handpicking. The clonal rat beta cell line INS-1E was provided by C. Wollheim. Human islets were cultured in complete CMRL-1066 (Invitrogen) medium at 5.5 mM glucose, mouse islets and INS-1E cells were cultured in complete RPMI-1640 medium at 11.1 mM glucose and HEK 293 cells were cultured in DMEM. All media included glutamate, 1% penicillin-streptomycin and 10% FBS (all from PAA). INS-1E medium was supplemented with 10 mM HEPES, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. Islets and INS-1E cells were exposed to complex diabetogenic conditions: 22.2 or 33.3 mM glucose, 0.5 mM palmitic acid or the mixture of 2 ng/ml recombinant human IL-1β (R&D Systems, Minneapolis, MN) plus 1,000 U/ml recombinant human IFN-γ (PeproTech) for 72 h, 100 μM H_2O_2 for 6 h, 1 mM streptozotocin (STZ) for 8 h or 1 mM thapsigargin for 6 h (all Sigma). In some experiments, cells were additionally cultured with 10–25 μM JNK-selective inhibitor SP600125, 10 μM PI3K-selective inhibitor LY294002, 10 or 20 μM AKT inhibitor V, triciribine, a selective AKT1/2/3 inhibitor, 50 μM pancaspase inhibitor Z-VAD (OMe)-fmk, 100 μM Bax-inhibiting peptide V5 or Bax-inhibiting peptide, negative control, 20 μM InSolution MG-132, proteasome inhibitor (all Calbiochem), 100 nM glucagon like-peptide 1 (GLP1), 100 nM recombinant human insulin and 50 μg/ml cycloheximide (CHX) and 1 μM glibenclamide (all Sigma). Palmitic acid was dissolved as described previously[62].

Mice. For MLD-STZ experiments, 8- to 10-week old *Mst1*$^{-/-}$ mice on a 129/sv genetic background[55] and their *Mst1*$^{+/+}$ WT littermates were i.p. injected with STZ (40 mg per kg body weight; Sigma) freshly dissolved in 50 mM sodium citrate buffer (pH 4.5) or citrate buffer as control for five consecutive days (referred to as multiple low-dose (MLD)-STZ). To create beta cell–specific *Mst1*$^{-/-}$ mice, mice harboring exon 4 of the *Mst1* gene flanked by *loxP* sites (*Mst1*$^{fl/fl}$)[55] were crossed with mice expressing Cre under the rat insulin-2 promoter (B6;D2-Tg(Ins-Cre)23Herr: RIP-Cre[63], provided by P. Herrera, University of Geneva and A. Mansouri, Max Planck Institute for Biophysical Chemistry). RIP-Cre-*Mst1*$^{fl/-}$ mice were intercrossed to generate RIP-Cre-*Mst1*$^{fl/fl}$. Mice were MLD-STZ injected as described above. For the high-fat diet (HFD) experiments, 8-week-old RIP-Cre-*Mst1*$^{fl/fl}$ (b*Mst1*$^{-/-}$) mice and their RIP-Cre littermates were fed a normal diet (ND, Harlan Teklad Rodent Diet 8604, containing 12.2, 57.6 and 30.2% calories from fat, carbohydrate and protein, respectively) or a high-fat, high-sucrose diet (HFD, Surwit Research Diets, New Brunswick, NJ, containing 58, 26 and 16% calories from fat, carbohydrate and protein, respectively[58,64]) for 20 weeks. For both models, random blood was obtained from the tail vein of nonfasted mice, and glucose was measured using a Glucometer (Freestyle; TheraSense, Alameda, CA). Mice were killed at the end of experiment, and pancreases were isolated. Throughout the whole study, food consumption and body weight were measured weekly. Only male mice were used in the experiments. All animals were housed in a temperature-controlled room with a 12-h light-dark cycle and were allowed free access to food and water in agreement with US National Institutes of Health animal care guidelines and the German animal protection law and approved by the Bremen Senate.

Intraperitoneal glucose and ITTs and measurement of insulin release. For i.p. GTTs, mice were fasted 12 h overnight and injected i.p. with glucose (40%; B. Braun, Melsungen, Germany) at a dose of 1 g per kg body weight. Blood samples were obtained at time points 0, 15, 30, 60, 90 and 120 min for glucose measurements using a glucometer and at time points 0, 15 and 30 min for measurement of serum insulin levels. For i.p. ITTs, mice were injected with 0.75 U per kg body weight recombinant human insulin (Novolin, Novo Nordisk) after a 5-h fast, and glucose concentration was determined with the glucometer. Insulin secretion was measured before (0 min) and after (15 and 30 min) i.p. injection of glucose (2 g per kg body weight) and measured using ultrasensitive mouse Elisa kit (ALPCO Diagnostics, Salem, NH).

Plasmids. pCMV-Myc-MST1 and kinase-dead pCMV-MST1-K59R (dn-MST1) was provided by J. Sadoshima and Y. Maejima (Rutgers New Jersey Medical School)[30]. Mouse pB.RSV.PDX1-GFP plasmid was provided by I. Leibiger (Karolinska University, Stockholm). pcDNA3 Myr-HA AKT1, pCDNA3 HA-ubiquitin and pCDNA3 Jnk1a1 (apf) (dn-JNK) plasmids were obtained from Addgene (Cambridge, MA). Mouse PDX1 mutants (T11, T126, T152, T155, T214 and T231) in the pCGIG5 vector were generated by site-directed mutagenesis as described previously[38]. All mutations were verified by sequencing. To make bacterial expression plasmids for PDX1 mutants, the complete mouse PDX1 coding sequence (WT and mutants) has been amplified by PCR using a specific set of primers from pCGIG5 plasmids and cloned into a pGEX-6P-1 bacterial expression vector (provided by R. Walther, University of Greifswald). The rat insulin–driven luciferase vector (RIP-luc) was constructed by subcloning a 700–base pair (bp) fragment containing 660 bp of the rat insulin-2 promoter (provided by R. Zinkernagel, University of Zurich) into a pMCS-Green-*Renilla*-Luc vector (Thermo Scientific). pCMV-Red Firefly Luc vector was obtained from Thermo Scientific.

Transfections. To knock down MST1 expression in human islets, SMARTpool technology from Dharmacon was used. A mix of ON-TARGETplus siRNAs directed against human MST1 sequences UAAAGAGACCGGCCAGAUU, GAUGGGCACUGUCCGAGUA, GCCCUCAUGUAGUCAAAUA and CCA GAGCUAUGGUCAGAUA and mouse Mst1 sequences GAUGGGCACUG UCCGAGUA, UGACAGCCCUCACGUAGUC, GCAGGUCAACUUACAG AUA and CUACAGCACCCGUUUGUUA. (100 nM, Dharmacon) was transiently transfected into human and mouse islets and efficiently reduced MST1 levels. An ON-TARGETplus nontargeting siRNA pool from Dharmacon served as a control. To knock down BIM and caspase-3 expression in human islets, siRNAs targeting human BIM (SignalSilence Bim SiRNA I, Cell Signaling) and caspase-3 (NEB) were used. GFP, MST1, dn-MST1 (K59R), dn-JNK1 and Myr-AKT1 plasmids were used to overexpress these proteins in human islets and INS-1E cells.

An adapted improved protocol to achieve silencing and overexpression in human islets was developed[19,65]. Islets were partially dispersed with accutase (PAA) to break islets into smaller cell aggregates to increase transfection efficiency and cultured on ECM dishes for at least 2 d. Isolated islets and INS-1E cells were exposed to transfection Ca^{2+}-KRH medium (KCl 4.74 mM, KH_2PO_4 1.19 mM, $MgCl_2$•$6H_2O$ 1.19 mM, NaCl 119 mM, $CaCl_2$ 2.54 mM, $NaHCO_3$ 25 mM and HEPES 10 mM). After 1-h incubation, lipoplexes (Lipofectamine 2000, Invitrogen)/siRNA ratio 1:20 pmol or –lipoplexes/DNA ratio 2.5:1) were added to transfect the islets and INS-1E cells. After an additional 6-h incubation, CMRL-1066 or RPMI-1640 medium containing 20% FCS and L-glutamine were added to the transfected islets or INS-1E cells. Efficient transfection was evaluated based on fluorescein-labeled siRNA (NEB) or eGFP-positive cells analyzed by fluorescent or confocal microscopy. HEK 293 cells were transiently transfected using Opti-MEM medium and Lipofectamine (Invitrogen) according to the manufacturer's instructions.

Glucose-stimulated insulin secretion. For acute insulin release in response to glucose, primary human and mouse islets and INS-1E cells were washed and preincubated (30 min) in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose and 0.5% BSA. KRB was then replaced by KRB 2.8 mM glucose for 1 h (basal), followed by an additional 1 h in KRB 16.7 mM glucose with or without 100 nM GLP1 or 1 μM glibenclamide. Insulin content was extracted with 0.18N HCl in 70% ethanol. Insulin was determined using human and mouse insulin ELISA (ALPCO Diagnostics, Salem, NH). Secreted insulin was normalized to insulin content.

Immunohistochemistry. Pancreatic tissues were processed as previously described[66]. In brief, mouse pancreases were dissected and fixed in 4%

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formaldehyde at 4 °C for 12 h before embedding in paraffin. Human and mouse 4-μm sections were deparaffinized, rehydrated and incubated overnight at 4 °C with anti-insulin (A0546, 1:50), anti-glucagon (A0565, 1:50) and rat anti–mouse Ki-67 (M7249, 1:50) antibodies from Dako, anti-pMST1 (3681, 1:100) and anti-Bim (2933, 1:100) antibodies from Cell Signaling Technology (CST), anti-PDX1 antibody (47267, 1:1,200) from Abcam and anti-Glut2 antibody (07-1402, 1:100) from Chemicon, followed by FITC- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, 1:100). Slides were mounted with Vectashield with DAPI (Vector Labs). For mouse sections or primary islets cultured on ECM dishes, beta cell apoptosis was analyzed by the TUNEL technique according to the manufacturer's instructions (*In situ* Cell Death Detection Kit, TMR red; Roche) and double stained for insulin. Fluorescence was analyzed using a Nikon MEA53200 (Nikon, Dusseldorf, Germany) microscope, and images were acquired using NIS-Elements software (Nikon).

Morphometric analysis. For morphometric data, ten sections (spanning the width of the pancreas) per mouse were analyzed. Pancreatic tissue area and insulin-positive area were determined by computer-assisted measurements using a Nikon MEA53200 (Nikon, Dusseldorf, Germany) microscope, and images were acquired using NIS-Elements software (Nikon). The number of islets (defined as insulin-positive aggregates at least 25 μm in diameter) was scored and used to calculate islet density (number of islets per square centimeter of tissue), mean islet size (the ratio of the total insulin-positive area to the total islet number on the sections). Mean percentage beta cell fraction per pancreas was calculated as the ratio of insulin-positive to whole pancreatic tissue area. Beta cell mass was obtained by multiplying the beta cell fraction by the weight of the pancreas. Morphometric beta cell and islet characterizations are results from analyses of at least 100 islets per mouse.

Western blot analysis. At the end of the incubation periods, islets and INS-1E cells were washed in ice-cold PBS and lysed in lysis buffer containing 20 mM Tris acetate, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1% Triton X-100, 5 mM sodium pyrophosphate and 10 mM β-glycerophosphate. Prior to use, the lysis buffer was supplemented with Protease and Phosphatase Inhibitors (Pierce, Rockford, IL, USA). Protein concentrations were determined with the BCA protein assay (Pierce). Equivalent amounts of protein from each treatment group were run on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electrically transferred onto PVDF membranes. After 1-h blocking at room temperature using 5% milk (CST), membranes were incubated overnight at 4 °C with the following antibodies: rabbit anti-MST1 (3682), rabbit anti-pMST1 (3681), rabbit anti-ubiquitin (3933), rabbit anti-Bim (2933), rabbit anti-AKT (9272), rabbit anti-pAKT (Ser437) (4058), rabbit anti-Bax (2772), rabbit anti–Bcl-2 (2870), rabbit anti–Bcl-xL (2764), rabbit anti-Bad (9239), rabbit anti-pBad (5284), rabbit anti-PUMA (4976), rabbit anti-Bak (6947), rabbit anti-Mcl1 (4572), rabbit anti-pan–phosphorylated threonine (9381), mouse monoclonal anti-pan–phosphorylated threonine (9386), rabbit anti-pGSK-3 (9336), rabbit anti-pFOXO1 (9461), mouse anti-Myc (2276), rabbit anti–cleaved caspase-3 (9664), rabbit anti–cleaved caspase-9 (rat specific; 9507), rabbit anti–cleaved caspase-9 (human specific; 9505), rabbit anti–cytochrome *c* (4272), rabbit anti–cytochrome oxidase (4850), rabbit anti-pJNK (T183/Y185) (9251), rabbit anti–c-Jun (S63) (9261), rabbit anti-PARP (9542), rabbit anti-tubulin (2146), rabbit anti–glyceraldehyde 3-phosphate dehydrogenase (2118) and rabbit anti–β-actin (4967) all from CST); rabbit anti-pMST1 (79199), rabbit anti-GFP (290), mouse anti-NOXA (13654) and rabbit anti-PDX1 (47267) all from Abcam; rabbit anti-pH2B (07-191) from Millipore and rabbit anti-pPDX1 (T11) (PA5-13046) from Thermo Scientific, followed by horseradish-peroxidase–linked anti-rabbit or anti-mouse IgG (Jackson). All primary antibodies were used at 1:1,000 dilution in Tris-buffered saline plus Tween-20 (TBS-T) containing 5% BSA, except for the antibody to PDX1 (1:6,000). Membrane was developed using a chemiluminescence assay system (Pierce) and analyzed using DocITLS image acquisition 6.6a (UVP BioImaging Systems, Upland, CA, USA).

Immunoprecipitation. For immunoprecipitation, cells were washed with PBS and lysed in cold buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1% NP-40, 5 mM sodium pyrophosphate and 10 mM β-glycerophosphate supplemented with proteinase and phosphatase inhibitors for 30 min on ice. Lysates were centrifuged at 12,000*g* for 15 min at 4 °C before immunoprecipitation. Immunoprecipitations were carried out with incubating 0.5–1 mg of total lysate with rabbit anti-PDX1 (1:500; Abcam), rabbit anti-MST1 (1:50; CST), mouse anti-Myc (1:1,000; CST) and rabbit anti-GFP (1:1,000; Abcam) antibodies on a rotator at 4 °C overnight. Immunocomplexes were then captured with Protein A Agarose Fast Flow (Millipore) by rotation at 4 °C for 4 h. After five washes with cold lysis buffer, the immunoprecipitates were used for kinase assays or resuspended in sample buffer and separated by NuPAGE 4–12% Bis-Tris gels (Invitrogen).

***In vitro* kinase assay.** Purified human active MST1 without GST-tag (Upstate Biotechnology) or with GST-tag (Abcam) was incubated with ^{32}P-ATP (2 μCi, PerkinElmer Life Sciences), ATP (100 μM) and 1 mM dithiothreitol in a kinase buffer containing 40 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 1 mM EDTA and 1 μg of purified recombinant human PDX1 (Abcam) or bacterially purified PDX1-GST (WT and mutants) as substrates. After incubation at 30 °C for 30 min, the reaction was stopped by adding loading buffer and proteins were separated on NuPAGE gels and phosphorylation levels visualized either by autoradiography or specific antibody for pPDX1 (T11) or pThr. The total PDX1 was detected with anti-PDX1 antibody.

***In vivo* kinase assay.** HEK 293 cells were transiently transfected with PDX1 and MST1 expression plasmids. Next, cell lysates were subjected to immunoprecipitation with anti-PDX1 antibody. The immunoprecipitates were separated by NuPAGE Bis-Tris gels and transferred to PVDF membranes and subsequently subjected to analyses of phosphorylation levels by pan–phosphorylated threonine antibody, which binds to threonine phosphorylated sites in a manner largely independent of the surrounding amino acid sequence or pan–phosphorylated serine antibody, which recognizes serine-phosphorylated proteins.

***In vivo* ubiquitination.** HEK 293 cells were cultured in 10-cm cell culture dishes and transfected with HA-ubiquitin and PDX1 and MST1 expression plasmids for 48 h. For ubiquitination in human islets, 5,000 islets per condition were transfected with ubiquitin plasmid. After 24 h, islets were infected with Ad-GFP or Ad-MST1 for 6 h and kept for another 48 h. HEK 293 cells and islets were exposed to 20 μM MG-132 for the last 6 h of the experiment. Lysates were immunoprecipitated with PDX1-specific antibody overnight at 4 °C. Immunocomplexes were then captured with Protein A Agarose by rotation at 4 °C for 4 h. After extensive washing, immunoprecipitates were boiled in sample buffer and proteins subjected to western blotting with ubiquitin-specific antibody.

Protein degradation analysis. HEK 293 cells were transfected with PDX1 alone or together with MST1 expression plasmids. Human islets were infected with Ad-GFP (control) or Ad-MST1. At 48 h after transfection or infection, cells were treated with 50 μg/ml translation initiation inhibitor CHX, which was added to the medium at the times indicated, and the lysates were subjected to western blotting.

RNA extraction and RT-PCR analysis. Total RNA was isolated from cultured human islets and INS-1E cells using TRIzol (Invitrogen), and RT-PCR was performed as described previously[67]. For analysis, we used the Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, CA, USA) with TaqMan(R) Fast Universal PCR Master Mix for TaqMan assays (Applied Biosystems). TaqMan(R) Gene Expression Assays were used for *PDX1* (Hs00426216_m1), *SLC2A2* (Hs01096905_m1), *GCK* (Hs01564555_m1), *INS* (Hs02741908_m1), *PPIA* (Hs99999904_m1), *BCL2L11* (Hs01083836_m1) and *TUBULIN* (Hs00362387_m1) for human and *Pdx1* (Rn00755591_m1), *Slc2a2* (Rn00563565_m1), *Gck* (Rn00688285_m1), *Ins1* (Rn02121433_g1), *Ins2* (Rn01774648_g1), *Ppia* (Rn00690933_m1) and *Tuba1a* (Rn01532518_g1) for rat.

Luciferase reporter assay. The transcriptional activity of the PDX1 at promoter level was evaluated using rat insulin-2–*Renilla* luciferase (Ins2–luc *Renilla*) reporter gene. HEK 293 cells were transfected with Ins2–luc *Renilla*,

pCMV-firefly, PDX1-WT or PDX1-T11A, alone or together with Myc-MST1 expression plasmids for 48 h. INS-1E cells transfected with Ins2-luc *Renilla* and pCMV-firefly plasmids and were infected with Ad-GFP or Ad-MST1 for 48 h. Luciferase activity was determined using the *Renilla* Firefly Luciferase Dual Assay Kit according to the manufacturer's instructions (Pierce). pCMV-firefly was used as transfection control.

Adenovirus infection. Isolated human islets and INS-1E cells were infected with adenovirus carrying eGFP as a control (provided by A.E. Karlsen, Novo Nordisk A/S, Denmark) or MST1 (Ad-MST1, provided by J. Sadoshima) at a multiplicity of infection (MOI) of 20 (for INS-1E) or 100 (for human islets) for 4 h. Adenovirus was subsequently washed off with PBS and replaced by fresh medium with 10% FBS, and GSIS or RNA and protein isolation performed after 48 or 72 h after infection.

Purification of PDX1-GST recombinant proteins. Expression and induction of recombinant GST proteins were performed as described previously[68]. *Escherichia coli* BL21 cells with various GST-fusion expression plasmids were cultured at 37 °C, and expression of recombinant proteins was induced by 0.1 mM final concentration of isopropyl-β-D-thiogalactoside (IPTG; Sigma) for 2.5 h. Cells were lysed using B-PER bacterial protein extraction reagent (Pierce) and purified using Glutathione Spin Columns (Pierce).

Cytochrome *c* release. Cytochrome *c* release was performed by digitonin-based subcellular fractionation technique[69]. Briefly, INS-1E cells were digitonin-permeabilized for 5 min on ice after resuspension of the cell pellet in 200 µl of cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 (pH 7.2) with 300 µg/ml digitonin (Sigma). Cells were then centrifuged at 1,000*g* for 5 min at 4 °C. Supernatants (cytosolic fractions) were collected and pellets solubilized in the same volume of mitochondrial lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100 and 0.3% NP-40), followed by centrifugation at 10,000*g* for 10 min at 4 C. After centrifugation, supernatants, which are the heavy membrane fractions enriched for mitochondria, as well as cytosolic fractions were subjected to western blot analysis.

Generation of stably expressed shRNAMST1 INS1-E cell line. To knock down MST1 expression in INS-1E cells, two different lentiviral shRNA targeting MST1 or control shRNA vectors (pGIPZ collection, Open Biosystems, Huntsville, AL) were transfected into INS-1E cells, and stable clones were generated by selection with puromycin (1 to 2.5 µg/ml). Positive clonal cell lines were identified by immunoblotting using antibody directed against MST1. After selection, INS-1E lines were maintained in culture medium containing 1.5 µg/ml puromycin.

Statistical analyses. Samples were evaluated in a randomized manner by five investigators (A.A., V.K., S.K., T.Y. and Z.A.) who were blinded to the treatment conditions. To perform statistical analysis, at least 3 independent experiments from 3 different organ donors were performed for human islets, at least 3 independent experiments were performed for mouse islets and cell lines and at least 3 independent tissue samples or mice were included in the analyses, as reported in all figure legends. No statistical method was used to predetermine sample size. Data are presented as means ± s.e.m. Mean differences were determined by Student's *t*-tests. To account for multiplicity in the treated cells *in vitro* and in mice *in vivo*, a Bonferroni correction was used.

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