Modulating the Hippo Pathway as Novel Strategy for β-Cell-Directed Therapy in Diabetes

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Summary

Diabetes with its chronic hyperglycaemia and severe metabolic disturbance became a serious health problem world-wide. In type 1 diabetes (T1D), the loss of β-cell mass is associated with immune mediated destruction, whereas type 2 diabetes (T2D) is initially triggered by insulin resistance and thus a higher insulin demand from the β-cells. Both diseases are characterised by apoptosis of pancreatic β-cells. Identification of key regulatory mechanisms and active signaling pathways involved in β-cell failure in diabetes is crucial to further complete the current knowledge of disease pathogenesis and is necessary for future strategies aiming to prevent β-cell destruction in diabetes. The Hippo pathway is the key intracellular pathway, responsible for cell proliferation and apoptosis. Several lines of evidence attest the importance of mammalian Sterile-20-like kinases 1 (MST1), a critical Hippo pathway component, and its upstream activators Ph domain and leucine rich repeat protein phosphatases 1 and 2 (PHLPP1/2) in the regulation of β-cell survival and function.

In the first part of my thesis, I identified a small molecule inhibitor neratinib, a well-known irreversible pan-HER/EGFR tyrosine kinase inhibitor, as potent MST1 inhibitor. Neratinib significantly improves β-cell survival and function in several experimental models of diabetes in *in vitro* and *ex vivo* settings and restores functional β-cell mass *in vivo* in mouse models of both autoimmune associated T1D and obesity associated T2D.

In the second part of my thesis, investigation of β-cells under diabetic conditions shows that PHLPPs are highly upregulated and can promote β-cell dysfunction and death. PHLPP1 and PHLPP2 are highly upregulated in human islets isolated from organ donors with T2D as well as from obese diabetic mouse models. The apoptosis inducing effects of PHLPPs in β-cells are mediated not only through the inactivation of pro-survival AKT signaling but also through the activation of pro-apoptotic MST1 kinase, two well-established PHLPP substrates, constituting a regulatory triangle loop to control β-cell survival. I identified chronic hyper-activation of mammalian target of rapamycin complex 1 *(*mTORC1) as primary mechanism of PHLPPs upregulation linking metabolic stress to β-cell death. Pharmacological and genetic inhibition of mTORC1 signaling reduces PHLPPs' protein levels and β-cell apoptosis, suggesting an mTORC1-dependent regulation of PHLPPs. Additionally, systemic PHLPP1 inhibition in PHLPP1-knockout mice improves glucose tolerance and insulin secretion, and promotes β-cell survival, proliferation and compensatory β-cell mass expansion in T1D and T2D mouse models of diabetes. Consistently, siRNA-mediated knockdown of PHLPPs restores β-cell function and survival in human islets isolated from donors with T2D *ex vivo*. All these results indicate the important role of PHLPP1/2 in diabetes progression, which may serve as potential targets for drug development.

Taken together, my findings identify important mechanisms of β-cell failure in diabetes and demonstrate the functional significance of targeting MST1 and its upstream activators PHLPPs in restoring pancreatic β-cell mass in diabetes.

Zusammenfassung

Diabetes einhergehend mit chronischen Hyperglykämien und schweren Stoffwech--selstörungen ist weltweit zu einem ernsten Gesundheitsproblem geworden. Im Typ-1-Diabetes (T1D) ist der Verlust der β-Zellmasse mit einer immunvermittelten Zerstörung verbunden, während Typ-2-Diabetes (T2D) zunächst durch Insulinresistenz und damit einem höheren Insulinbedarf ausgelöst wird. Beide Krankheiten sind durch Apoptose der β-Zellen des Pankreas gekennzeichnet. Die Identifizierung der wichtigsten regulatorischen Mechanismen und aktiven Signalwege, die am Versagen der β-Zellen im Diabetes beteiligt sind, ist von entscheidender Bedeutung für die Pathogenese der Krankheit und insbesondere für künftige Strategien, die darauf abzielen, die Zerstörung der β-Zellen und damit Diabetes zu verhindern. Der Hippo-Signalweg ist maßgeblich verantwortlich für Zellproliferation und Apoptose. Es gibt mehrere Belege für die Bedeutung der Mammalian-Sterile-20-ähnlichen Kinase 1 (MST1), einer kritischen Komponente des Hippo-SignalWegs, und ihrer vorgeschalteten Aktivatoren, der Ph-Domäne und Leucin-reichen Repeat-Proteinphosphatasen 1 und 2 (PHLPP1/2) bei der Regulierung des Überlebens und der Funktion der β-Zellen.

Im ersten Teil meiner Dissertation habe ich einen niedermolekularen Inhibitor, Neratinib, einen irreversiblen pan-HER/EGFR-Tyrosinkinaseinhibitor, als potenten MST1-Inhibitor identifiziert. Neratinib verbessert maßgeblich Überleben und Funktion von β-Zellen in verschiedenen experimentellen Modellen des Diabetes in *in vitro* und *ex vivo* und stellt die funktionelle β-Zellmasse *in vivo* in Mausmodellen von sowohl autoimmunassoziiertem T1D als auch fettleibigkeitsassoziiertem T2D wieder her.

Im zweiten Teil meiner Dissertation zeigen Untersuchungen von β-Zellen unter diabetischen Bedingungen, dass PHLPPs stark hochreguliert sind und die Dysfunktion und den Tod von β-Zellen fördern können. PHLPP1 und PHLPP2 sind in menschlichen Inselzellen, die aus Organspendern mit T2D isoliert wurden, sowie in adipösen diabetischen Mausmodellen stark hochreguliert. Die apoptoseinduzierenden Wirkungen von PHLPPs in β-Zellen werden nicht nur durch die Inaktivierung der überlebensfördernden AKT-Signalübertragung vermittelt, sondern auch durch die Aktivierung der pro-apoptotischen MST1-Kinase, zweier bekannter PHLPP-Substrate, die ein regulatorisches Dreieck zur Kontrolle des β-Zellüberlebens bilden. Ich habe eine chronische Hyperaktivierung des Mammalian Target of Rapamycin Komplex 1 (mTORC1) als primären Mechanismus der PHLPP-Hochregulierung identifiziert, der metabolischen Stress mit dem Tod von β-Zellen verbindet. Die pharmakologische und genetische Hemmung der mTORC1-Signalübertragung führt zu einer Verringerung der PHLPPs-Proteine und Zellapoptose, was auf eine mTORC1-abhängige Regulierung von PHLPPs hindeutet. Darüber hinaus verbessert die systemische PHLPP1-Hemmung bei

PHLPP1-Knockout-Mäusen die Glukosetoleranz und Insulinsekretion und fördert Überleben, Proliferation und die kompensatorische Vermehrung der β-Zellmasse in T1D- und T2D-Mausmodellen. Folgerichtig stellt die siRNA-vermittelte Ausschaltung von PHLPPs die Zellfunktion und das Überleben in menschlichen Inseln wieder her, die von Spendern mit T2D *ex vivo* isoliert wurden. All diese Ergebnisse deuten auf eine wichtige Rolle von PHLPP1/2 bei der Diabetesprogression hin, die als potenzielle Angriffspunkte für die Arzneimittelentwicklung dienen.

Zusammenfassend identifiziert meine Arbeit einen wichtigen Mechanismus des β-Zellversagens im Diabetes und zeigt die funktionelle Bedeutung einer gezielten Beeinflussung von MST1 und seiner vorgelagerten Aktivatoren PHLPPs bei der Wiederherstellung der pankreatischen β-Zellmasse im Diabetes.

List of Abbreviations

4E-BP	eukaryotic translation initiation factor 4E-binding protein
AICAR	5-Aminoimidazole-4-carboxamide riboside
AMOT	angiomotin
AMPK	AMP-activated protein kinase
APCs	Antigen presenting cells
ATG	autophagy-related
BAK	Bcl-2-antagonist/killer
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BIM	Bcl-2 like protein 11
CMA	Chaperone-mediated autophagy
eEF2K	elongation factor 2 kinase
DEPTOR	DEP domain-containing mTOR interacting protein
EGFR	Epidermal growth factor receptor
eIF4B	protein synthesis initiation factor 4B
FFA	Free fatty acids
FKBP12	FK506-binding protein 12
FOXM1	Forkhead box M1
GCK	Glucokinase
GLUT2	Glucose transporter 2
GPCR	G protein-coupled receptors
Grb	Growth-factor-bound protein
GSIS	Glucose-stimulated insulin secretion
GSK	Glycogen synthase kinase
hIAPP	Human islet amyloid polypeptide
IFN	Interferon
IL-1	Interleukin 1
INSR	insulin receptor
IRS	insulin receptor substrate
JNK	c-Jun NH2-terminal kinase
KCTD	Potassium channel tetramerization domain containing
KIBRA	kidney and brain protein
LAT	L-type amino acid transporter
LATS	Large tumor suppressor
LC3	Microtubule-associated protein 1A/1B-light chain 3
MafA	MAF BZIP Transcription Factor A
MAP4K	Mitogen activated protein kinase kinase kinase kinase
MLD	multiple low-dose
mLST	mammalian lethal with Sec13 protein
MOB	MOB kinase activator
MST	Mammalian sterile-20-like kinases
mTOR	mammalian/mechanistic target of rapamycin
mSin	Mitogen-activated-protein-kinase-associated protein
NeuroD1	Neurogenic Differentiation 1
NF2	Neurofibromatosis 2

NF-β	Nuclear factor kappa β
NGN	Neurogenin
NKX6.1	Homeobox protein Nkx-6.1
NLK	Nemo-like kinases
PARP	Poly ADP ribose polymerase
PDGFR	platelet-derived growth factor receptor
PDK	phosphoinositide-dependent kinase
PDX1	Pancreatic and Duodenal Homeobox 1
PHLPP	PH domain and leucine rich repeat protein phosphatase
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-biphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PP2C	protein phosphatase 2C
PPAR	peroxisome proliferator-activated receptor
PPM	metal-dependent protein phosphatase
PRAS40	Proline rich AKT substrate of 40kDa
Rag	Ras-related GTPase
RASSF	ras association domain family member
Rheb	Ras homolog enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RUNX	Runt-related transcription factor
S6K	Ribosomal protein S6 kinase
SAV	Salvador
SGK	Serum- and glucocorticoid-induced protein kinase
SREBP	sterol regulatory element binding protein
STAT	signal transducer and activator of transcription
STK	Serine/Threonine Kinase
STZ	Streptozotocin
TAZ	transcriptional coactivator with PDZ-binding motif
TAO	thousand and one amino acid protein
TEAD	TEA domain
TFEB/E3	Transcription factor EB/E3
TNF	Tumor necrosis factor
TNFR	TNF receptor
TSC	Tuberous sclerosis complex
ULK	Unc-51 like kinase
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
YAP	Yes-associated protein

Table of Content

1 Introduction

1.1 Diabetes - Silent epidemic is becoming public health emergency

Diabetes mellitus is a group of metabolic diseases, which can be simply described as systemic disorder of glucose homeostasis leading to multiple anatomical and functional abnormalities. The far most common form is type 2 diabetes (T2D), with an estimated 90% occurrence, whereas the majority of the remaining 10% have type 1 diabetes (T1D) and some other rarer types such as gestational diabetes during pregnancy and maturity onset diabetes of the young (MODY), and neonatal diabetes, both caused by a single gene mutation (1).

T2D is the most frequent metabolic disorder which has increased significantly in recent decades and becomes a major healthcare concern globally. T2D is thought to be highly provoked by diets involving excessive nutrient consumption especially in western countries although genetics is an important factor in individuals developing T2D (2). As such, diabetes is interdependently linked to obesity as better glycemic control and improved diabetic prognosis has been reported in patients with T2D who lost weight (3). Between 1980 and 2004, the global rise in obesity, sedentary lifestyles as well as ageing population have fourfold increased the incidence of T2D. Worryingly, since the T2D incidence continues to increase, it is projected that there will be more than 590 million patients diagnosed with this condition by 2035 (4,5). Diabetes together with cardiovascular disease, cancer, and respiratory disease accounts for over 80% of all premature deaths. Apart from shorter life expectancy, patients with diabetes are at higher risk of getting infections, cardiovascular disease, stroke, end-stage kidney disease, chronic liver disease, and cancer (6) and suffer from more severe COVID-19 (7). Until 30 years ago, most of our knowledge on pancreatic islet morphology and the mechanisms leading to diabetes development came from very limited studies of human pancreatic islets and from animal models such as non-obese diabetic (NOD) mice, ob/ob and db/db mouse models of obesity and T2D, respectively. In the last few decades, pancreas tissue banks (e.g., the Network for Pancreatic Donors with Diabetes: nPOD and the Exeter Archival Diabetes Biobank), which are dedicated to the study of diabetes, were able to make an important progress in our understanding of pathophysiology of diabetes (8). We can now screen for many early disease biomarkers which are associated with increased risk of diabetes including high blood concentrations of pro-inflammatory cytokines (e.g., C-reactive protein, interleukin-6 (IL-6) and tumor necrosis factor (TNF)), low adiponectin levels, higher blood concentrations of branched-chain and aromatic amino acids and gut flora metabolites, and have therefore the ability to detect and start supportive therapy in early stages (9–12).

T1D is an autoimmune disease characterized by an absolute insulin deficiency due to selective destruction of β-cells mediated by T-lymphocytes and autoantibody production. Activated self-reactive lymphocytes infiltrate the pancreas, initiated by the release of proinflammatory cytokines, which activate intracellular signaling cascades of inflammatory and apoptotic signaling pathways. Although T1D onset is quite different from T2D, both disorders share the same key issue – β-cell apoptosis and loss of functional β-cell mass (13,14).

T2D is characterized by systemic insulin resistance as well as the loss of functional pancreatic β-cells, and impaired insulin secretion (15). Currently there are no available therapies targeting the cause of diabetes, namely the dying β-cells, only treatments for improved insulin secretion and decreased peripheral tissue insulin resistance are available (16,17). With that in mind better understanding of the molecular bases of diabetes and ultimately highly effective therapeutic approaches are urgently in need. Typically, T2D develops slowly over time with an initial phase of prediabetes. Insulin resistance in muscle, liver and adipose tissue and declining β-cell function are the early abnormalities with higher insulin levels progressing to early impairment of glucose tolerance (18). It has been reported that the decline in β-cell function can begin an average of 12 years before T2D is diagnosed (19). However, because of the higher demand for insulin, β-cells are trying to increase its production by expanding β-cell mass through β-cell proliferation and/or hypertrophy. Over time, when β-cells become exhausted and are unable to cope with insulin resistance and body's higher demand for insulin to promote normal metabolic homeostasis, β-cells undergo programmed cell death, called apoptosis (20–22). For graphical presentation of diabetes-associated β-cell death and its systemic effect please see Fig.1.

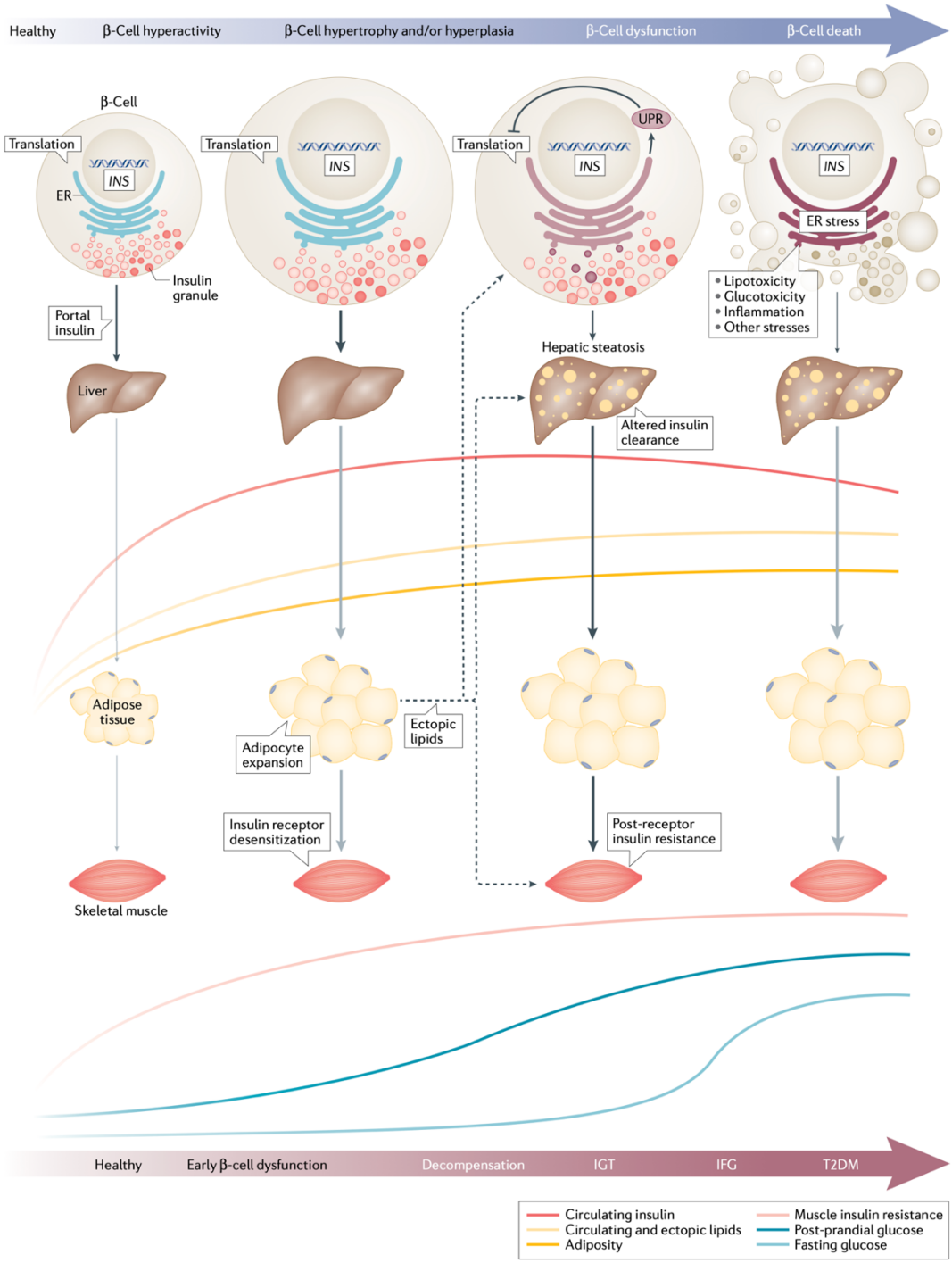

Figure 1: Metabolic stress in pancreatic β-cells is associated with β-cell death, hepatic steatosis, adiposity and insulin resistance. Glucotoxicity, lipotoxicity and inflammation during the development of pre-diabetes ultimately leads to β-cell hyperactivation. Such β-cell hyperactivation triggers adipocyte expansion, hepatic lipid accumulation and muscle and systemic insulin resistance. Insulin resistance increases demand for the insulin for better glucose clearance capacity causing β-cell hyperactivity and hypertrophy. Eventually, β-cells lose their ability to respond effectively to high metabolic stress leading to β-cells dysfunction and death, and the onset of type 2 diabetes. From (23).

1.2 Pathophysiology of β-cell in Type 2 Diabetes: Mediators and Mechanisms of Failure

Apoptosis is considered as a hallmark of diminished pancreatic β-cell mass in T2D as well as in T1D. Patients with T2D have up to 60 % reduced β-cell mass in pancreatic islets. However, as reported by Butler *et al.* underlying mechanism for reduction of β-cell mass is most likely a dramatic increase in the rate of β-cell apoptosis, which is apparent from the histological observations of the pancreatic islets from patients with T2D (21,24). There are two main apoptotic pathways in mammalian cells, extrinsic and intrinsic (extensively reviewed in (25)). Briefly, the extrinsic signaling pathway initiates apoptosis through transmembrane receptor-mediated interactions, which involve death receptors that are members of the TNF-family. Differently, the intrinsic signaling pathway does not require receptor-mediated stimuli but is instead triggered by intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. Despite all the discoveries, β-cell death as the final event of the apoptotic cascade has, up to date, not been entirely characterized.

1.2.1 Glucotoxicity and Lipotoxicity

Chronic exposure to high glucose, free fatty acids (FFAs), islet amyloid polypeptide (IAPP) and cytokines increase production of reactive oxygen species (ROS), induce endoplasmic reticulum (ER) stress, and disrupt autophagy in β-cells (Fig. 2). The later altogether affect β-cell metabolism leading to β-cell dysfunction and activation of signaling pathways of cell death, resulting in consequential reduction of β-cell mass (13,14,26). High glucose concentration causes dysfunction of the β-cells mainly by oxidative stress through the overproduction of free radicals by the electron transport system in mitochondria. This reduces the activity of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lowers concentrations of NADPH, which in turn leads to the reduction of β-cells' antioxidant capacity (27,28). The antioxidant defenses of pancreatic β-cell cannot cope with high ROS formation and the protective balance is lost in individuals with diabetes. In return, oxidative stress leads to cellular damage and β-cell dysfunction (29). Furthermore, β-cells are very vulnerable to oxidative stress also because the main antioxidant enzymes such as superoxide dismutase 1 and 2 (SOD1-2), glutathione peroxidase 1 (GPX1), and catalase (CAT) are expressed at low levels (30,31). Apart from the cell damage, the high oxidative load of radicals activates stress response pathways, such as nuclear transcription factor-kappa B (NF-κB) and JNK (c-jun N-terminal kinase) which promote β-cell death (32,33). Activation of JNK has been shown to play a critical role in β-cell apoptosis induced by glucotoxicity and ROS formation (34). Also, ER stress-overload in β-cells due to their high rates of proinsulin biosynthesis in response to glucose stimulation putts additional pressure on β-cell homeostasis and regulated insulin secretion. Signs of β-cell ER stress are

evident in *in vitro* and *ex vivo* studies with islets from T2D donors (35,36). Immunostaining and electron microscopy of β-cells from organ donors with T2D showed significant expansion and morphological changes of the ER with elevations in the unfolded protein response (UPR) with highly increased late-stage UPR-induced transcription factor C/EBP homologous protein (CHOP), promoting β-cell apoptosis in T2D and obesity (37–39). Moreover, proinsulin synthesis is highly prone to misfolding and is a major direct cause of physiological ER stress in β-cells, leading to chronic ER stress in diabetic β-cells due to misfolded proinsulin accumulation. Hyperproinsulinemia has been reported in both individuals with prediabetes and T2D (40). Diabetic β-cells have defective insulin processing even in the early stages of disease pathology (41). Wrongly processed proinsulin is secreted to the blood stream and its levels can reach up to 50% of total insulin in the circulation. Such incorrect processing and secretion of proinsulin by the remaining β-cells further contributes to the decreased insulin levels (23,42,43).

Similar to glucotoxicity, lipotoxicity also plays a fundamental role in β-cell dysfunction. It has been shown that FFAs potentiate β-cell death in the presence of high glucose concentration (44). Acute FFA or glucose overload in pancreatic β-cells increases insulin secretion, but a prolonged period of both causes inhibition of insulin secretion and of necessary genes for β-cell identity and promotes apoptosis (44). Moreover, saturated free fatty acid palmitate itself can directly inhibit key β-cell transcription factors Pancreatic and duodenal homeobox 1 (PDX1) and MAF BZIP Transcription Factor A (MafA), causing loss of β-cell identity (45). Lipotoxic condition is also considered as mediator of ER stress causing disruption in ER Ca^{2+} homeostasis and blocked protein folding (38). Notably, ROS formation due to excessive β-oxidation of the fatty acids puts additional pressure to antioxidant mechanisms of the β-cells similar to glucotoxicity (46).

1.2.2 Role of the Human Islet Amyloid Polypeptide

Under chronic high glucose and free fatty acid exposure, increased expression of human islet amyloid polypeptide (hIAPP) or amylin has been associated with the formation of pathophysiological amyloid deposits resulting in β-cell dysfunction and death (47,48). In human islets, amyloid aggregation develops in connection with diabetes and is observed in 90% of autopsy pancreata of T2D donors (49). Because of differences in the sequence, toxic oligomers of amyloidogenic proteins which form amyloid fibrils are absent in rodents (50). hIAPP is one of the most aggregation-prone peptide with irreversible aggregation process. Initially, hIAPP amyloid deposition is intracellular as a result of defective processing of pro-IAPP in the ER followed by the release of such misfolded IAPP molecules from the secretory

granule of β-cells in the extracellular matrix (ECM) (Fig. 2) (51) seen in patients with T2D (52,53). Amyloid deposits can occupy up to 80% of the islet extracellular space and disrupt the normal islet architecture between the capillaries and extracellular matrix. These anatomical changes affect the normal passage of glucose and hormones within the pancreatic islets and thereby lead to abnormalities in insulin secretion in T2D (54,55). In addition, fibril-shaped IAPP inserts itself into the cell membrane resulting in disruption of membrane structure causing membrane leakage (56). Increased disruption of autophagy has also been reported since hIAPP aggregates might damage the p62 dependent lysosomal degradation pathway (57).

1.2.3 Pancreatic Islet Inflammation in Type 2 Diabetes

Low-grade islet inflammation is a major feature of T2D. While for many decades islet immune cell infiltration of $CD4^+$ and $CD8^+$ T-cells are recognized as the leading cause of β-cell destruction of T1D, it now became clear that increased numbers of macrophages, $CD4^+$ T-cells, cytokines, and chemokines also participate in the destruction of pancreatic islets in T2D. Typically, there is a chronic, subclinical 2- to 3-fold systemic increase of proinflammatory cyto- and chemokines with increased number of macrophages (58–60). Interleukin-1β (IL-1β) is a master regulator and amplifier of immunological responses in human pancreatic islets and governs other cytokines, such as interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor α (TNFα), and chemokine response. Increased production and secretion of IL-1β is induced by glucotoxicity (61) with even higher potentiation in a combination with saturated FFAs (62,63). These glucose-induced effects could be mediated directly by acting on immune cells or via glucose-induced upregulation of the islet amyloid system, which is a strong activator of islet inflammation. In immune cells, IAPP triggers a strong proinflammatory response, including the secretion of IL-1β and release of various chemokines including CCL2, CCL3, CXCL1, CXCL2, and CXCL10 (59,64,65). In non-immune cells, hIAPP promotes additional release of chemokines which recruits additional macrophages to the islets and potentiate inflammation (48). Kamata *et al.* reported that pancreatic islets from patients with T2D showed increased levels of amyloid deposits and only those islets presented increased macrophages (66). Furthermore, a very recent study described another local activator of islet inflammation, coming from the β-cells itself. It suggests that local macrophages sense and monitor the microenvironment of β-cell secretory activity by reacting to ATP, which is co-secreted with insulin by β-cells. Sensing β-cell activity ensures that β-cells remain healthy and stable, and that any abnormal activity leads to macrophage activation and an inflammatory response (67).

1.2.4 Loss of β-cell Identity in Diabetes

Unbalanced rate of enhanced β-cell apoptosis and failure of β-cell proliferation is thought to be a main reason for reduced β-cell mass. But lately other proposed mechanisms such as dedifferentiation and/or transdifferentiation have also been introduced in the β-cell pathology of T2D. While normal physiological glucose stimulation is essential for the maintenance of the β-cell phenotype, persistent hyperglycemia not only induces apoptosis but can lead to the loss of β-cell identity through the loss of β-cell markers and expression of non-β-cell genes, causing β-cell reprograming to a less differentiated state (68–70). β-cell dedifferentiation is characterized by down-regulation of β-cell specific genes such as those encoding β-cell specific transcription factors, surface glucose transporters, and genes involved in glucose metabolism, insulin synthesis and insulin secretion (71). In addition, the endocrine precursor cell genes and some genes that are low in the expression of normal, mature β-cells are up-regulated as well (70,71). Such changes ultimately lead to the loss of primary β-cell characteristics and function. PDX1 plays a critical role in the regulation of β-cell fate by promoting genes essential for β-cell identity and repressing genes linked to α-cell identity (72). Beside PDX1, many other genes involved in the maturation and function of β-cells such as Insulin 1 (Ins1), Insulin 2 (Ins2), Glut2, MafA and NK6 Homeobox 1 (Nkx6.1), are reduced in pancreatic β-cells in T2D (73–77). β-cells may undergo dedifferentiation under certain pathophysiological conditions such as glucotoxicity due to oxidative stress. Reactive oxygen species accumulate in β-cells and prevent the expression of PDX1 and MafA, eventually resulting in reduced β-cell function (74,78–80). Pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα have also been shown to promote β-cell dedifferentiation in human and mouse islets (81).

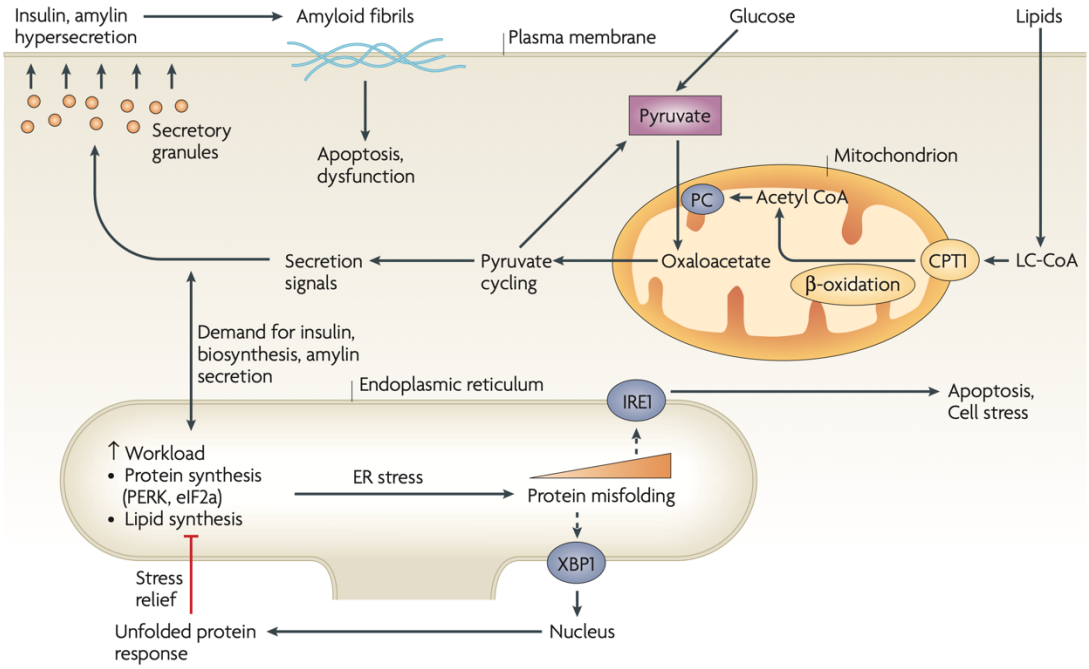

Figure 2: Overview of β-cell failure in type 2 diabetes. Nutritional overload and increased lipid supply induce enzymes of β-oxidation resulting in increased acetyl CoA levels and constitutive upregulation of pyruvate cycling. This leads to the insulin hypersecretion with abnormal glucose-stimulated insulin secretion. The increased demand for insulin biosynthesis increases the workload in the ER, gradually leading to ER stress and increased protein misfolding. Such extreme pressure on ER triggers the unfolded protein response (UPR), which fails under persistent ER stress resulting in β-cell apoptosis. Additionally, high insulin secretion is accompanied by amylin secretion, which in humans form amyloid fibrils that accumulate at the surface of β-cells inducing dysfunction and apoptosis. From (82).

1.3 Cellular Signaling Pathways Regulating β-cell Function, Survival and Apoptosis

1.3.1 mTOR Complex 1 and 2

The Basics. Through evolution organisms have developed mechanisms allowing them to survive and grow in environments where nutrient availability might not be consistent. Since growth and proliferation requires the consumption of considerable amounts of energy, cells are forced to sense and quickly react to the nutrient and energy availability to maintain cell growth. In mammals, an example of such mechanism is the protein kinase mTOR (mechanistic target of rapamycin) pathway. mTOR signaling controls and regulates various highly complex processes to translate large amounts of energy and nutrients into regulating basic cell functions such as growth and proliferation (83,84). Therefore, any dysregulation of mTOR may have a huge effect on cell behavior and is a common characteristic of many

diseases, including cancer, cardiovascular diseases and neurodegenerative processes as well as obesity and T2D (85–88).

More than 25 years ago, starting with the efforts to understand the mechanisms of the drug rapamycin, biochemical approaches led to the identification of the physical target of rapamycin, mTOR (89–91). Since then, our understanding of its complex mechanism of action is still evolving. mTOR is a serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and forms two distinct protein complexes: mTOR Complex 1 (mTORC1) and 2 (mTORC2). These are big complexes with mTORC1 having six and mTORC2 seven known associated proteins. They both share the mTOR subunit, as well as mLST8 (mammalian lethal with Sec13 protein 8), DEPTOR (DEP domain containing mTOR-interacting protein), and the Tti1/Tel2 complex. In addition, Raptor (regulatory protein associated with mTOR) and PRAS40 (proline-rich Akt substrate of 40 kDa) are mTORC1-specific whereas Rictor (rapamycin-insensitive companion of mTOR), mSin1 (mammalian stress-activated map kinase-interacting protein 1), and protor1/2 (protein observed with rictor 1 and 2) are only part of mTORC2 (83,92,93).

Rapamycin as an important immunosuppressant and anticancer drug forms a complex with FKBP12 (12-kDa FK506-binding protein) and binds to the FKBP12-rapamycin binding (FRB) domain of mTOR and obstructs substrates from entering the active site. While rapamycin-FKBP12 directly inhibits mTORC1, mTORC2 is characterized by its insensitivity to acute rapamycin treatment (89,94). Recent analysis of mTORC2 structures suggest that RICTOR blocks the FKBP12–rapamycin complex binding site on mTOR and thereby makes mTORC2 insensitive to acute inhibition by rapamycin. However, prolonged rapamycin treatment inhibits the assembly of mTORC2 and reduces the levels of mTORC2 below those needed to maintain normal AKT/PKB signaling (95,96).

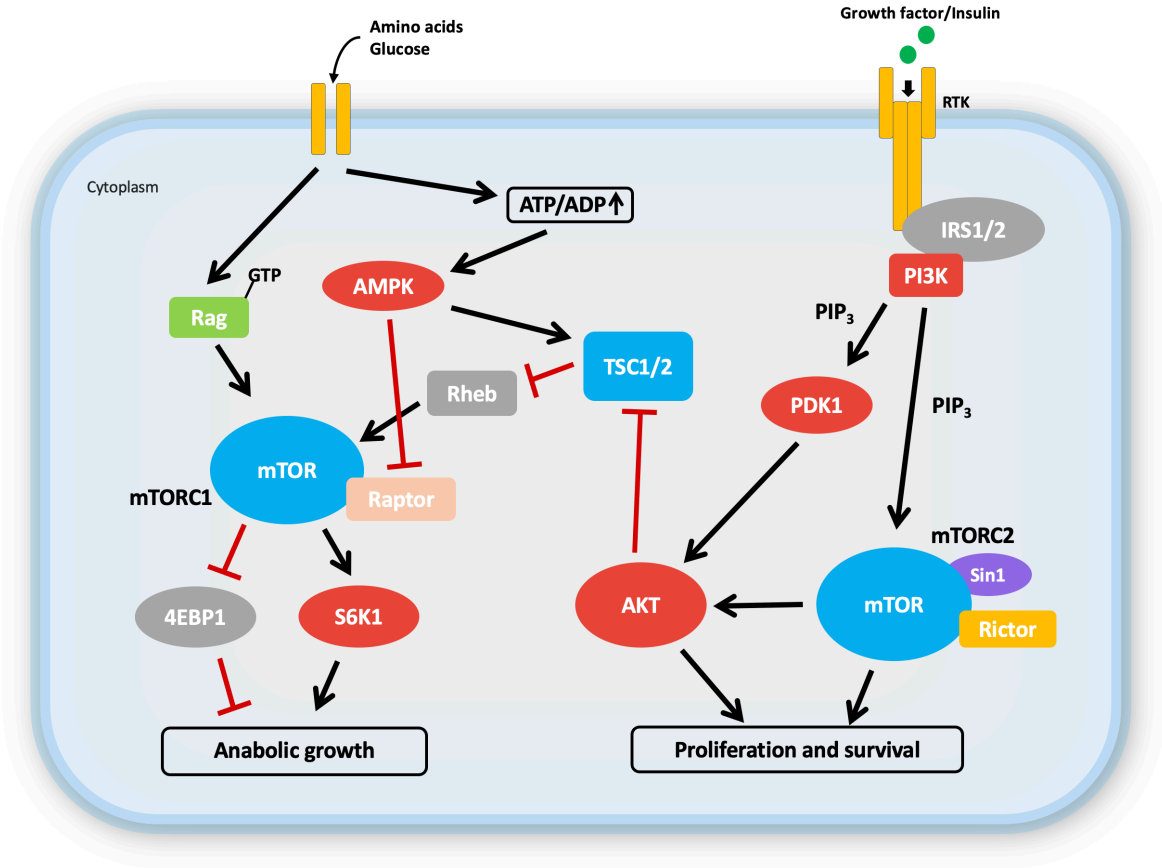

Figure 3: Regulators of mTOR signaling pathway. mTOR complex 1 (mTORC1) and mTORC2 sense upstream environmental information to precisely control metabolism in the cell. mTORC1 translocate to the lysosome in the presence of amino acids and/or glucose by the help of the Rag GTPases. Once located on the lysosomal surface, mTORC1 is additionally activated by the small GTPase Rheb. In response to cellular stimulation by growth factors/insulin, PI3K promotes the activation of mTORC2 and AKT. AKT further phosphorylates and inhibits the upstream inhibitor of mTORC1, known as TSC1/2. TSC1/2 acts as a "central switch" for mTORC1, sensing the signals from growth factors/insulin (through AKT) and cellular energy levels (through AMPK), and activates mTORC1 in the presence of favorable energy status. This leads to GTPase Rheb stimulation and mTORC1 activation. mTORC1 promotes anabolic growth by stimulating protein synthesis (through S6K and 4EBP), nucleotides, and lipid biosynthesis as well as inhibition of catabolic processes such as autophagy (through TFEB and ULK1). mTORC2 is mainly controlling cell survival and cell proliferation. AMPK, AMP-activated protein kinase; AKT/PKB, protein kinase B; IRS1/2, insulin receptor substrate 1 and 2; MST1, mammalian STE20-like kinase 1; mTORC1/ 2, mechanistic target of rapamycin complex 1 and 2; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PIP3, phosphotidylinositol (3,4,5)-trisphosphate; PKC, Protein kinase C; Rag, Ras-related GTPase; Rheb, Ras homolog enriched in brain; S6K1, ribosomal S6 kinase 1; SGK1, Serum/ Glucocorticoid Regulated Kinase 1; TFEB, transcription factor EB; TSC1/2, tuberous sclerosis protein complex 1 and 2; ULK1, UNC-5 like autophagy activating kinase 1 (inserted from our previous review article (85)).

1.3.2 mTOR Signaling Partners: Upstream and Downstream

mTORC1 Signaling. Nutrients, such as amino acids and glucose, as well as growth factors, oxygen and energy levels are the main activators of mTORC1. Based on these stimuli mTOR coordinates the synthesis of essential biomolecules, such as lipids, proteins, and nucleotides (92,97). They regulate mTORC1 through the heterodimeric Rag GTPases (98,99). Amino acid-dependent stimulation of mTORC1, where leucine and arginine are especially important, affects RagA/B-GTP vs. RagA/B-GDP ratio through GAP activity of GATOR1 (100) allowing them to bind Raptor and recruit mTORC1 to the lysosomal membrane, near to Rheb, for full activation (Fig. 3) (101). Growth factors activate another side of mTORC1 through small GTPase Rheb [97], which resides on the lysosomal membrane and directly stimulates mTOR kinase activity when it is bound to GTP. As a major depot for recycled biomolecules, the lysosome is an important organelle of regulation for mTORC1 signaling. It degrades macromolecules so that their elements can be reused as new building blocks within the cell (103). These macromolecules get into the lysosome through two major pathways – autophagy and endocytosis. However, in the absence of Rags, mTORC1 is not able to translocate to the surface of lysosome and stays inactive. Since mTORC1 activation requires activated Rags and Rheb, it is necessary that both, amino acid and growth factor signaling is functional (104). Tight coordination of these signals ensures that mTORC1 kinase activity is only turned on when all the requirements for the optimal cell growth are fulfilled.

Activated mTORC1 initiates protein synthesis by modulating mRNA translation machinery through phosphorylation of S6Ks (ribosomal protein S6 kinases) and 4E-BPs (eukaryotic initiation factor 4E binding proteins). mTORC1-orchestrated phosphorylation and activation of S6K further phosphorylates several substrates such as ribosomal protein S6 (a component of the 40S ribosomal subunit), eIF4B (protein synthesis initiation factor 4B), and eEF2K (elongation factor 2 kinase), which promote translation initiation and elongation (105). Besides, mTORC1 phosphorylation of translation inhibitor 4E-BP dissociates 4E-BP from the main initiation factor eukaryotic translation initiation factor 4E (eIF4E), which enables the formation of eIF4F complex responsible for cap-dependent translation (106). When cells increase in size new lipids must be generated to sustain biogenesis of new membranes. Thus, mTORC1 controls expression of numerous genes involved in fatty acid and cholesterol synthesis through S6K1-dependent mechanism by activating the major lipogenic regulator transcription factor SREBP1/2 (sterol regulatory element binding protein 1 and 2) and PPARγ (peroxisome proliferator-activated receptor γ) (107). When sterol levels are low, the SREBPs translocate from the ER membrane to the nucleus to boost de novo lipid and cholesterol synthesis (108). Another well-established function of mTORC1 is to promote the nucleotide

synthesis required for DNA replication and ribosome biogenesis in growing cells. It has been shown that mTORC1 activates transcription factor ATF4 and its downstream target MTHFD2 (mitochondrial tetrahydrofolate cycle enzyme methylene tetrahydrofolate dehydrogenase 2) to drive *de novo* purine synthesis (109). Additionally, CAD (carbamoyl-phosphate synthetase) is phosphorylated and activated by S6K1 and acts as a critical component of the *de novo* pyrimidine synthesis pathway (84,110). Autophagy, the key cellular survival mechanism during starvation, is also repressed by mTORC1 in order to prevent catabolism and direct all the energy towards anabolism and cell growth. It directly acts on the ULK (UNC-5-like autophagy activating kinase) complex required for the induction of autophagy and autophagosome formation by phosphorylating key complex components ULK1 and ATG13 (autophagy related gene 13). mTORC1 phosphorylation of ULK1 and ATG13 suppresses autophagy to prevent a futile cycle in which newly synthesized cellular building blocks are prematurely broken down (111). Indirectly, mTORC1 binds and phosphorylates TFEB (transcription factor EB) which maintains TFEB in the cytoplasm and prevents it from translocating to the nucleus. mTORC1-dependent phosphorylation of TFEB triggers interaction with 14-3-3 binding partner and prevents TFEB nuclear translocation where it activates gene expression for lysosomal biogenesis and autophagy (103,112). In contrast, prolonged starvation will shut down mTORC1 allowing induction of autophagy pathway and lysosome synthesis. Newly formed lysosomes then break down proteins and release essential monomers back to the cytoplasm to renew the pool of amino acids, enabling reactivation of the mTORC1 pathway (113). Since cell growth and cell proliferation are tightly linked it is not surprising that mTORC1 may also have an important role in cell-cycle progression as reported from several studies (114–117).

mTORC2-AKT Signaling. mTORC2, the 2nd axis of mTOR, is primarily activated by extracellular stimuli such as growth factors and insulin but recent studies demonstrated new possibilities of direct mTORC2 activation also by glucose and glutamine (118,119). Activation of PI3K (Phosphoinositide 3-kinases) by growth factors triggers activation and production of PIP3 (phosphatidylinositol-(3,4,5)-trisphosphate), which physically interacts with mSin1, a structural component of mTORC2, resulting in mTORC2 activation (Fig. 3) (120). Interestingly, recent findings suggests that mTORC2 may also be activated by direct phosphorylation by AMPK under energetic stress and starvation to promote cell survival (121). Activated mTORC2 is the main driver of cell proliferation and survival as well as cytoskeleton dynamics and organization by phosphorylating a subset of protein kinase A/protein kinase G/protein kinase C (AGC) family members such as AKT, SGK1 (serum- and glucocorticoid-regulated kinase 1), and PKC (protein kinase C) (84,105). Besides, mTORC2 also inhibits the hippo central kinase MST1 (mammalian sterile 20-like kinase 1), an important regulator of the

apoptosis pathway (122). mTORC2 dysregulation is linked to several pathological abnormalities such as cancer and T2D (120).

mTORC2-mediated AKT phosphorylation at the hydrophobic motif of S473 allows PDK1 (3-phosphoinositide-dependent protein kinase 1) to further phosphorylate AKT at T308 for its full activation to promote cell survival and proliferation. These two sites are obligatory for the proper activation of AKT (123). Activated AKT acts as central player of growth factor-mediated mTORC1 activation forming a major signaling node between mTORC1 and mTORC2 through the following signaling pathways (83,124): 1. AKT-mediated phosphorylation and inactivation of TSC2 (tuberous sclerosis complex protein 2) forms a heterodimeric complex with TSC1 leading to its dissociation from the lysosome and its substrate Rheb allowing mTORC1 to be recruited near Rheb and activated (102). 2. AKT-induced inhibition of glycogen synthase kinase 3 (GSK3) also leads to reduction in TSC1/2 activation and consequently to the induction of mTORC1. 3. AKT-operated direct phosphorylation and inhibition of PRAS40, an inhibitor of mTORC1, which also leads to mTORC1 activation. On the contrary, mTORC1 inhibits mTORC2-AKT signaling axis through multiple regulatory feedback loops (Fig. 4). There are few different loops of mTORC1 involving IRS1/2 (insulin receptor substrate 1 and 2) and mTORC2 main components Rictor and Sin1, extensively described and discussed in the context of metabolism in our recent review paper (106). In principle, these regulatory feedback mechanisms serve as physiological ‘‘brake’’ to turn off excessive growth factor-stimulated activation of PI3K-mTORC2-AKT signaling. Studies of AKT signaling in recent years using sophisticated biochemical approaches have revealed the complexity of its cellular signaling network (120). One of the pivotal targets of AKT are FoxO (the forkhead box O) transcription factors which control numerous highly important genes for apoptosis, cell proliferation and growth and metabolism. FoxO is also involved in the adaptation to fasting and low insulin and IGF1 signaling. AKT-triggered phosphorylation of FoxO leads to the translocation of FoxO out of the nucleus and cytosolic sequestration by 14-3-3 proteins, therefore terminating their transcriptional program.

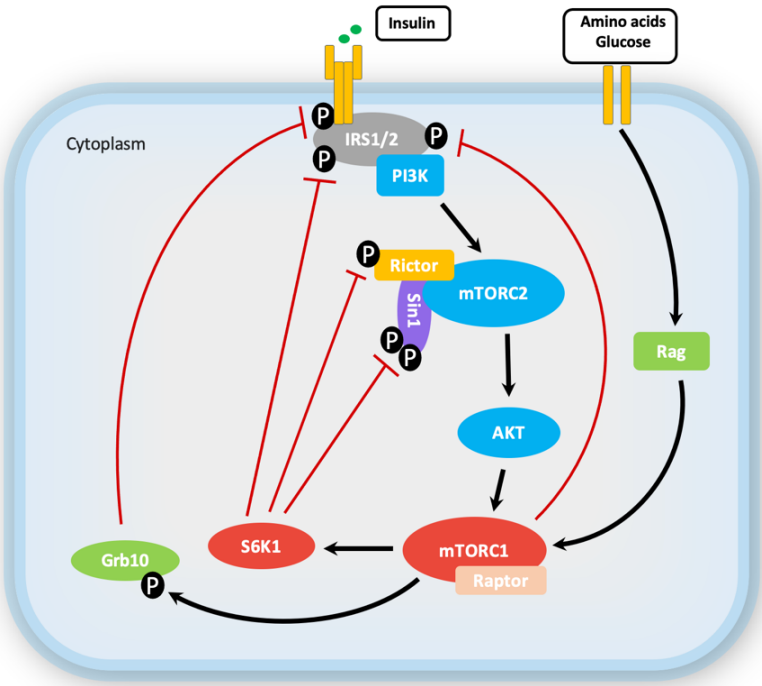

Figure 4: mTORC1-S6K1-mediated negative feedback loops. mTORC1 controls several negative feedback loops as intracellular protective mechanisms regulating cell growth and homeostasis by restricting excessive actions of RTK-IRS1/2-PI3K and mTORC2 signaling. AKT/PKB, protein kinase B; Grb10, growth-factor-bound protein 10; IRS1/2, insulin receptor substrate 1 and 2; mTORC1/2, mechanistic target of rapamycin complex 1 and 2; PI3K, phosphoinositide 3-kinase; Rag, Ras-related GTPase; Raptor, regulatory-associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; S6K1, ribosomal S6 kinase 1 (inserted from our previous review article (85)).

1.3.3 mTOR Dysregulation in Pancreatic Islets in T2D

mTORC1 Hyperactivation in T2D. After an initial phase of functional adaptation and compensation in response to insulin resistance and metabolic demand, β-cells gradually lose their ability to regulate glucose homeostasis due to constant β-cell overwork and progressively, T2D develops. As we formulated in our recent review “metabolic pathways and related molecular mechanisms responsible for β-cell damage during nutritional overload are not clearly understood. mTORC1 plays a key role in nutrient sensing and integration of metabolic and nutritional stimuli to regulate cellular metabolism, survival and growth. While physiological mTORC1 activation is essential and beneficial for the maintenance of normal β-cell homeostasis and insulin secretion, development of inappropriate hyperactivation of mTORC1 leads to β-cell failure“ (106). Sustained mTORC1 activation showed improved glucose homeostasis and hyperinsulinemia as well as enhanced β-cell hypertrophy and hyperplasia in young mice. In contrast, old mice with prolonged mTORC1 hyperactivation

developed hyperglycemia and β-cell failure possibly due to the inhibition of autophagy as protective mechanism of β-cells making them more prone to apoptosis (106,125,126). T2D, obesity, and insulin resistance have been linked to changes in serum amino acids, especially with elevations in branched-chain and aromatic amino acids. Branched-chain amino acids, primarily leucine, are highly effective activators of mTORC1 and may boost its activity during T2D (127). Hyperactivation of mTORC1 together with the reduction of mTORC2 activity, mainly due to negative feedback loops, has been seen in mouse models of T2D as well as in human islets isolated from T2D donors (128,129). Moreover, chronic hyperactivation of mTORC1 progresses to hyperglycemia and diminished β-cell mass in β-cell specific TSC2 knockout mice ($\beta TSC2^{-/-}$) (130). Such loss in β-cell function and mass is due to apoptosis caused by ER overload and impairment in autophagy with an excessive accumulation of damaged mitochondria (131). Hyperactivated mTORC1 is the central trigger of ER stress in β-cells by enhancing protein synthesis and therefore increasing the protein load within the ER. Glucolipotoxicity is known to trigger β-cell death through ER stress in human and rodent β-cells due to the mTORC1-mediated upregulation of IRE1 (part of UPR), which induces apoptosis through activation of ASK1 and JNK (132,133). As reported, mTORC1 inhibition is beneficial for β-cell function and glucose homeostasis in Akita mice, a model for ER stress-induced diabetes (134). In addition, inhibition of mTORC1 signaling improves β-cell function and insulin secretion and restores mTORC2 activity in islets isolated from patients with T2D as well as from mouse models of T2D (128,135). mTORC2 has been studied less extensively in β-cells than mTORC1 but appears to have critical roles in β-cell adaptation to metabolic stress, β-cell proliferation, survival, and glucose-stimulated insulin secretion (124,136,137). Reduction in mTORC2 activity is primarily caused by the negative regulation of hyperactivated mTORC1 which phosphorylates the mTORC2 subunits mSin1 and Rictor leading to declined pro-survival mTORC2-AKT signaling (106). Therefore, all these studies point to mTORC1 as a signaling hub that is hyperactivated during nutrient excess and diabetes progression and because of the accelerated metabolism, important control functions are lost; with β-cell failure as the final outcome.

Lysosomal Dysfunction in T2D. Lysosomes are the terminal effectors of the autophagy pathway which break down macromolecules allowing β-cells to clean up defective organelles and molecules (138). Lysosomal damage and dysfunction are characterized by lysosomal membrane permeabilization (LMP) primarily caused by oxidative stress. Following LMP, proteolytic hydrolases are released into the cytoplasm where they activate caspases and other proapoptotic proteins to trigger cell death (139). Lysosomal biogenesis is mainly regulated by transcription factors EB (TFEB) and E3 (TFE3) (140), with TFEB also regulating several genes for autophagy machinery initiation (141). Lysosomal biogenesis is largely a response to

nutrient starvation and stressors to initiates degradative processes to restore cellular homeostasis. Prolonged starvation, ER stress or mitochondrial damage translocate TFEB and TFE3 to the nucleus and therefore initiate lysosomal biogenesis to allow cells to cope with increased nutrient demand through catabolic processes and to “clean” itself and remove damaged organelles (142). In contrary, under the nutrient overload when mTORC1 is constitutively active it prevents lysosomal biogenesis by phosphorylating and disrupting TFEB stability and its cytoplasmic to nuclear shuttling. Accordingly, TFEB is less likely to be present in the nucleus in diabetic β-cells exposed to chronic nutrient overload with hyperactive mTORC1 (142,143). Islets from patients with T2D and human islets exposed to glucolipotoxic conditions show an accumulation of autophagosomes within their cytosol (144,145). This may at least in part happen due to lysosomal dysfunction during T2D and glucolipotoxicity, as dysfunctional lysosomes reduce autophagosome clearance, leading to autophagosome accumulation (146).

Impaired Autophagic Flux in T2D. The lysosomal system is tightly linked with autophagy to maintain a healthy intracellular environment and protect β-cell function during nutrient stress with three major types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (all comprehensively reviewed in (147)). Besides, crinophagy has been described as another important subclass of autophagy possessed by secretory cells such as pancreatic β-cells. Crinophagy is an energy-efficient method to eliminate excess secretory granules to prevent an overaccumulation of insulin granules in β-cells when not needed (138,148). The most extensively studied subtype of autophagy in β-cells is macroautophagy (149). In brief, macroautophagy is a process that sequesters the macromolecules or other cytoplasmic components in a double membrane vesicle called autophagosome. The formation of autophagosome occurs through a series of events, which includes initiation, nucleation, elongation and maturation of a phagophore regulated by numerous kinases. Autophagosome then fuses with lysosomes and forms functional autophagolysosome (150). Impaired autophagic flux is one of the main causes for autophagic dysfunction arising from insufficient initiation of autophagosome formation or defects in autophagosome-lysosome fusion. Accumulation of dysfunctional organelles, protein aggregates, or metabolites together with the accumulation of autophagosomes (145) or autophagy proteins such as LC3-II and p62 (29,151,152) has been reported in β-cells isolated from T2D donors and in several T2D mouse models (143,153,154). All this indicates largely inhibited and defective autophagic flux in diabetic islets. Genetic deletion of Atg7 (autophagy-related gene 7), a critical protein in autophagy pathway, decreases the autophagy and impairs β-cell proliferation, survival, and insulin secretion as well as increases β-cell vulnerability (155). Moreover, mTORC1 hyperactivity in pancreatic β-cells promotes an impairment in the

autophagosome-lysosome fusion and increased accumulation of degenerated mitochondria (131). Exposing human and rodent islets to acute lipotoxicity induces autophagy to fight against ER stress-induced β-cell death (156,157). In contrast, long term lipotoxicity appears to impair autophagic flux in islets possibly due to hyperactivated mTORC1 (151,158,159). Likewise, in human islets sustained mTORC1 activation in response to glucolipotoxicity suppresses β-cell autophagy, leading to ER stress (134,158). Such ER stress can be prevented through suppression of mTORC1 and stimulation of autophagy (134,160–162). Additionally, continuous overnutrition in β-cells seen in metabolic disorders such as T2D inhibits AMPK, leading to chronic mTORC1 activation and thereby impairing autophagy flux (106,163). During starvation when AMPK is active, it phosphorylates critical autophagy-initiating kinase ULK1 at S317 and S777 and induces autophagy. In contrary, when nutrients are abundant mTORC1 phosphorylates ULK1 at S757 and inhibits autophagy by disrupting the interaction between ULK1 and AMPK and thus preventing autophagy initiation (164,165). Pharmacological stimulation of autophagy using molecules that activate AMPK, such as metformin, is the first-line pharmacological treatment of T2D (166) in a perfect scenario together with diet and lifestyle intervention. Defects in TFEB activity also contribute to autophagic dysfunction, as TFEB regulates numerous genes for autophagy initiation in addition to those for lysosomal biogenesis (141). Generally, changes in mTORC1 activity cause dysregulation of autophagy and eventually disrupt β-cell function and survival, demonstrating the important role of autophagy during nutrient stress.

1.4 The Hippo pathway

An Overview. Any tissue damage requires the activation of regenerative processes to restore normal organ function. First discovered using genetic screens in Drosophila and subsequently established in mammals, the Hippo pathway plays a fundamental role in organ growth and regeneration. A novel concept of how such mechanisms are controlled inside the cell had been identified. Cell-cell junctions, intrinsic and extrinsic mechanical force, cell density, energy stress, cell polarity, actin cytoskeleton dynamics, growth factors and other stimuli all regulate the Hippo pathway (167–169). In a very classical way, the Hippo pathway includes a kinase cascade with core components MST1/2 (mammalian STE-like 1 and 2; *Drosophila* homolog Hpo), and LATS1/2 (large tumor suppressor 1 and 2; *Drosophila homolog* Wts) kinases with regulatory subunits SAV1 and MOB1A/B, respectively. These kinases further control two transcriptional co-regulators: YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) (167,168). In response to cellular or environmental stress, active MST1/2 phosphorylate and activate LATS1/2, which in turn phosphorylate and

suppress YAP/TAZ (Fig. 5). This then promotes their cytoplasmic localization through binding to 14-3-3 proteins and their proteasomal degradation through recruitment of the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase (Hippo pathway "ON") (170,171). YAP/TAZ that have not been phosphorylated enter the nucleus to drive gene expression. When Hippo is „OFF", YAP/TAZ that have not been phosphorylated and remain in the nucleus to drive gene expression through interactions with DNA-binding transcription partners, the most important of which are the TEAD1-4 (TEA domain) transcription factors as well as others (e.g. SMADs and RUNX) (172,173). These transcription factors are crucial to maintain proper cell growth, proliferation and tissue homeostasis (174–176).

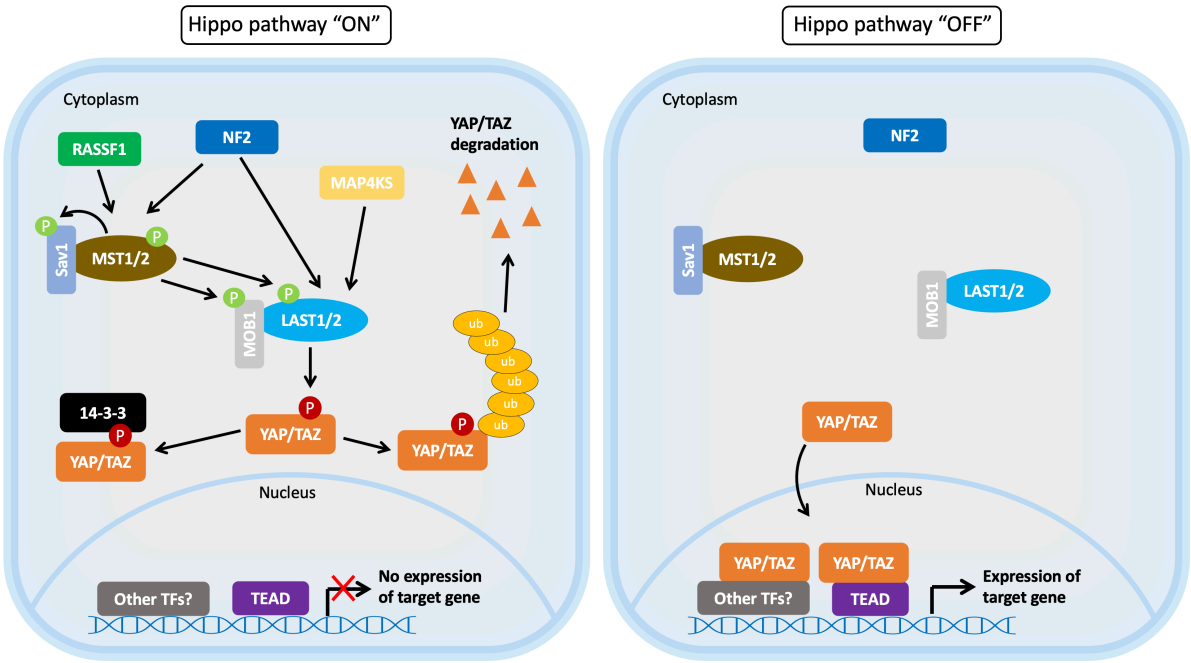

Figure 5: The Hippo pathway and its regulation. The Hippo pathway integrates multiple cellular and extracellular inputs such as cell junctions, cell polarity and mechanical forces to regulate the gene expression and organ growth. Hippo core components include MST1/2, LATS1/2, SAV1, MOB1, MAP4K4, and Merlin/NF2, as well as terminal effectors YAP, TAZ, and TEAD. Activated Hippo pathway initiates the phosphorylation of MST1/2 by NF2 and RASSF1, followed by LATS1/2 phosphorylation. Activated LATS1/2 further induce the phosphorylation of YAP/TAZ to promote their cytoplasmic sequestration and/or proteasomal degradation. When the Hippo pathway is not active, YAP/TAZ translocates and accumulates in the nucleus, bind to the TEAD transcription factors and promote the expression of target genes. NF2, neurofibromin 2/Merlin; MST1/2, Ste20-like kinase 1/2; LATS1/2, large tumor suppressor kinase 1/2; YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; MAP4K, mitogen-activated protein kinase kinase kinase kinase; RASSF1, ras association domain family member 1. SAV1, Salvador 1; MOB1, MOB kinase activator 1 (inserted from our previous review article (177)).

The MST1/2-LATS1/2-YAP/TAZ axis was the 1st and most established one in Hippo signaling, recent studies propose that the Hippo pathway is controlled by a complex upstream regulatory network including MAP4Ks (mitogen-activated protein kinase kinase kinase kinases), RASSFs (Ras-association domain-containing family proteins), KIBRA (kidney and brain protein), GPCRs (G-protein coupled receptors), TAO1–3 (thousand and one amino acid protein) kinases, MARKs (MAP/microtubule affinity-regulating kinases), the angiomotin family (AMOT, AMOTL1, and AMOTL2), and NF2 (neurofibromin 2, also known as Merlin) (Fig. 5) (167,168). MAP4K kinases can directly phosphorylate and activate LATS1/2 in the absence of MST1/2 while NF2 is activated by forming a complex with either KIBRA or AMOT to bind and recruit LATS1/2 to the plasma membrane where they are activated by MST1/2 kinases (178,179). TAO1 has been reported to phosphorylate and activate MST1/2 (180) but a recent study suggested that TAO kinases can also act as an activator of LATS1/2 (181). In addition, LATS1/2 activity can also be regulated by AJUBA protein, which inhibits LATS1/2 activity and activates YAP/TAZ suggesting LAT1/2-independent regulation of YAP/TAZ (182). Besides, there are also numerous other mechanisms regulating YAP/TAZ activity independent of LATS1/2. NDR1/2 (nuclear Dbf2-related) kinases, closely related to LATS1/2 kinases, can phosphorylate and inhibit YAP/TAZ at the same sites as LATS1/2 kinases. Moreover, NLK (Nemo-like kinases) and CDK7 can phosphorylate serine residues close to the LATS1/2 phosphorylation site to stabilize YAP/TAZ nuclear localization and activation (183,184). YAP/TAZ activity can also be affected by other mechanisms besides phosphorylation, including methylation by methyltransferases (185,186).

1.4.1 The Hippo Pathway in Cellular Metabolism

Several lines of new evidence demonstrate that the Hippo pathway can also be regulated by many metabolic pathways such as glycolysis, hexosamine biosynthesis, and mevalonate synthesis, as well as by master nutrient-sensing pathways, AMPK and mTOR, as described in our recent review (187). Glucose metabolism itself can increase YAP/TAZ binding to TEAD transcription factors leading to YAP/TAZ-dependent transcription activation and cellular growth. Specifically, PFK1 (phosphofructokinase 1), a key enzyme in glycolysis pathway, was described as a novel TEAD interacting partner, which regulates YAP/TAZ–TEAD complex formation (188). Similarly, hexosamine biosynthesis pathway (HBP), an alternative pathway of glucose metabolism, is also involved in Hippo pathway regulation. YAP/TAZ is O-GlcNAcylated by a main enzyme of the HBP, O-GlcNAc transferase, in the presence of elevated glucose levels, which stabilizes YAP/TAZ and can thus freely enter the nucleus and induces cell growth (189,190). Mevalonate-YAP/TAZ axis is another important regulator of Hippo pathway. Mevalonate pathway synthesizes steroid isoprenoids such as cholesterol, and

is involved in the protein prenylation, an important protein post-translational modification. It regulates Rho protein prenylation, which is required for proper activation of Rho GTPases that, in turn, activates YAP/TAZ and promotes their nuclear accumulation (191). In addition, the Hippo pathway is also largely regulated by one of its important upstream regulators, GPCR signaling. GPCRs are the largest and most diverse family of plasma membrane receptors regulating a wide array of physiological functions at normal and disease state. GPCR signaling can act as an activator or inhibitor of the Hippo pathway kinase LATS1/2. LPA (lysophosphatidic acid) and S1P (sphingosine 1-phosphophate) stimulates $G\alpha_{12/13}$ receptors, acting through Rho GTPases to inhibit the Hippo pathway kinases LATS1/2 and promote YAP/TAZ nuclear localization and activation. On the contrary, stimulation of $G\alpha_s$-coupled receptors by glucagon or epinephrine inhibits YAP/TAZ by activating LATS1/2 (192). Besides being regulated by metabolic signals, Hippo pathway is also an important regulator of metabolism. Recent findings characterized YAP/TAZ as an important modulator of glycolysis (193,194), gluconeogenesis (195) as well as nucleotide biosynthesis (196). Taken together this shows the complexity of the Hippo pathway in terms of its diverse responses to the metabolic cues. Such molecular responses are critical to ensure normal cellular and systemic metabolism since dysregulation of Hippo signaling importantly contribute to the pathogenesis of metabolic diseases, such as T2D, cardiovascular diseases and cancer.

1.4.2 The Hippo Pathway in Pancreatic Islets Development and Homeostasis

The Hippo pathway and its effectors have an important role in organ development, including heart, lung, brain, liver and pancreas (177,197–201). The importance of the Hippo pathway in pancreatic β-cells has been extensively reviewed in (177). In the developing pancreas endocrine cells differentiate from multipotent pancreatic progenitors (MPPs) that express Ngn3 (Neurogenin 3), a transcription factor essential for endocrine specification (202). Differentiation and maturation of pancreatic β-cells during the development is a second stage process driven by a complex network of numerous transcription factors such as PDX1, Neurod1, MafA, Nkx6.1, and many others (203–205). PDX1 in addition of being implicated in pancreatic development, plays a major role in keeping a healthy β-cell pool in adults (206). Because of these dominant roles, PDX1 is often termed as a “master regulator” of the whole β-cell development and function. Initial β-cell precursors possess high proliferation rate with proliferation as high as 4%. As they reach mature function, human β-cells lose their high initial proliferative rate to <0.05% at adolescence; only rodent β-cells still maintain the proliferative ability under specific conditions of high metabolic demand, such as high fat diet (207–209).

As one the most important downstream effectors of Hippo signaling, YAP/TAZ coordinate high proliferation rate during the initial stages of pancreas development but during cell-type specification it becomes limited to exocrine part and is switched off in the endocrine part to stop proliferation and boost the differentiation of mature and functional β-cells (177). In the mature pancreas YAP/TAZ expression is restricted to the exocrine ductal and acinar region and YAP is not detected, while TAZ shows very low detection within human and rodent pancreatic islets (210–214). Loss of YAP/TAZ is necessary to boost normal endocrine cell differentiation; YAP is characterized as “disallowed gene” with highest suppression in β-cells compared to other endocrine cell types (215). Interestingly, TAZ but not YAP may still be detected in adult pancreatic islets at a very low expression (214,216). The important question which remains is what are the mechanisms blocking YAP/TAZ expression? Even though not much is known there may be few potential explanations. Initially, the onset of Ngn3 expression during endocrine cell differentiation is adequate to suppress YAP/TAZ in developing pancreatic islets (217). Although Ngn3 levels decline after birth and are rarely detectable in the mature pancreas, YAP/TAZ levels do not reappear, suggesting more complex regulatory mechanisms (218). It is possible that YAP/TAZ expression in β-cells is turned off at the RNA level through microRNA regulation or by epigenetic regulation (210). Zhang *et al.* showed that YAP1 gene is a target of miR-375, which acts on YAP1 mRNA to reduce its mRNA and protein levels (219). Nevertheless, downstream transcription factors of the mammalian Hippo pathway, TEADs, remain active. TEAD1 has a central function in β-cells and is required for maintenance of mature β-cell function as well as β-cell quiescence (205,220). In principle, mature pancreatic β-cells are quiescent cells with an extremely low rate of proliferation and regenerative capability. Despite decades of research, we are still unable to completely understand the molecular mechanisms and underlying principles behind the quiescence of β-cells. Physiologically, it protects from massive uncontrolled insulin production, which would - at a worst scenario- lead to hypoglycemia and death. Nonetheless, β-cell quiescence could be a target for diabetes therapy. As recently suggested, mature β-cells maintain quiescence through direct transcriptional control of the cell cycle inhibitor *Cdkn2a* ($p16^{INK4A}$) by TEAD1. *Cdkn2a* permits proliferation of immature β-cells, but restricts proliferation of adult β-cells (216). TEAD1 also acts as a direct transcriptional activator of genes necessary for β-cells function, including *PDX1, Nkx6.1, MafA* and others, keeping β-cells healthy and functional (213). Regulators of TEAD1’s action in β-cells remain widely unknown. While factors that regulate the upstream Hippo kinase cascade could certainly play a role, other stimuli may also regulate TEAD1 function, independent of the Hippo pathway. Recent reports even suggest that TEAD1 might be controlled by palmitoylation and even glucose itself, which could affect TEAD1 localization and activity in β-cells (221–223).

1.4.3 Hippo Pathway: Dysregulation in Diabetic β-cells

The Hippo signaling is a dynamic pathway providing harmonised balance between proliferation, apoptosis, and differentiation and is mandatory for a precise formation and maintenance of pancreatic β-cells. Deregulated Hippo signaling can lead to pancreatic complications and defects in early embryogenesis and postnatal development and may trigger signalling cascades that initiate the expression of apoptotic genes in pancreatic β-cells resulting in β-cell destruction (177). To understand Hippo signaling in islet β-cell failure in diabetes, it is necessary to fully analyze the complex processes where dysregulation of single Hippo components can disrupt normal β-cell homeostasis. Some of the known mechanisms, which drive abnormal Hippo pathway activation and cause β-cell failure under diabetic conditions are described in this work. Initially, Ardestani *et al.* identified that the Hippo core kinase MST1 phosphorylates PDX1 at T11 site leading to its ubiquitination and degradation thereby causing defective β-cell function in rodent and human islets under diabetic conditions (224). In principle, MST1 is activated by two distinct mechanisms in stressed β-cells: i) an autophosphorylation of T183 site which leads to enzyme activation and ii) caspase-mediated cleavage of MST1 which generate a highly active truncated form of MST1. Both MST1-autophosphorylation as well as caspase-mediated cleavage of MST1 elevated in clonal β-cell under diabetogenic conditions as well as in isolated islets from diabetic mice and in human islets isolated from T2D donors. Activated MST1 integrates signals from pro-apoptotic diabetogenic stimuli to the mitochondrial-dependent apoptosis pathway by upregulating mitochondrial BCL-2 family member BIM and subsequent activation of caspase machinery i.e., caspase-3 cleavage and subsequent β-cell death. Through a positive feedback loop, cleaved caspase-3 can further cleave MST1 and boost the apoptotic response (225). Such internal activation loop between cleaved caspase 3, the main executioner of apoptosis, and cleaved MST1 potentiate apoptotic cascade under chronic nutrient or inflammatory stress, leading to β-cell failure. MST1 knockdown in human islets and INS-1 β-cells *in vitro* improves β-cell survival and function under pro-inflammatory cytokines and glucolipotoxic conditions. β-cell protective effect of genetic inhibition of MST1 was also confirmed *in vivo* in systemic MST1-knockout (KO) as well as in β-cell-specific MST1-KO mice (β-MST1-KO) under treatment with the diabetogenic compound streptozotocin (STZ). In addition, high fat high sucrose diet (HFD) induced hyperglycemia and β-cell failure is prevented in β-MST1-KO mice. Deletion of MST1 also greatly restores PDX1 protein expression in β-cells under stressful conditions (224,226). Consistently, Faizah *et al.* further confirmed the beneficial effect of MST1 inhibition on glycemia and glucose tolerance using small molecule MST1 inhibitor XMU-MP-1 in the STZ model of β-cell destruction and diabetes (227). Inhibition of MST1 by asialo-erythropoietin (asialo-rhuEPO^{P}) also protects rat RIN-m5F β-cell line from staurosporine-

induced apoptosis by suppressing MST1 and caspase-3 activation with improved PDX1 and insulin levels (228). High restoration of PDX1 levels and inactivation of MST1 was also demonstrated by GLP-1 analogue liraglutide after exposure to glucolipotoxicity (229) as well as in β-cells in HFD-induced diabetic mice (230).

Stress-induced MST1 activation acts as crucial modulator of apoptosis in β-cells but its regulation depends on many other Hippo kinases such as JNK, LATS1/2 and NF2/Merlin. JNK is a downstream target of MST1 and acts as central mediator of MST1-induced apoptosis triggering BIM-dependent mitochondrial apoptotic pathway (231). However, JNK can also act as upstream activator of MST1 through MST1 phosphorylation at S82 (232). Further, NF2 initiates Hippo signaling by either direct activation of the MST1, which further activates LATS1/2 or by direct recruitment of LATS1/2 to plasma membrane where it is activated by MST1 (179). As recently reported by us, hippo kinase LATS2 is also activated under diabetogenic conditions in both rodent and human islets/ β-cells. LATS2 hyper-activation induces β-cell failure through increased β-cell apoptosis and impaired β-cell function. Genetic silencing of LATS2 prevents β-cell apoptosis in clonal β-cell and isolated primary mouse and human islets *in vitro* and its genetic deficiency specifically in β-cells improves glycemia, insulin secretion and β-cell mass in mouse models of diabetes (151). Mechanistically, we identified LATS2 as key upstream activator of mTORC1 in stressed pancreatic β-cells, suggesting that the pro-apoptotic action of LATS2 is maintained via mTORC1. Such Hippo-mTORC1-autophagy crosstalk is described in detail in the next chapter.

1.5 Crosstalk between Hippo and mTOR Pathways

Hippo and mTOR are two dominant growth-control pathways that precisely dictate and control cell growth and homeostasis. These two highly conserved signaling pathways are extensively involved in physiological processes such as apoptosis, cell proliferation, cell differentiation and metabolism, and govern the pathogenesis of many human diseases. This explains the fact that these two signaling pathways have been highly conserved throughout the evolution. Therefore, it is rational to think that there must be a potential link and crosstalk between these two functionally similar pathways to accurately coordinate the cell number and size for proper organ growth and body development. Dysregulation of either pathways leads to tissue overgrowth (83,233–237). Emerging recent studies suggest that these two pathways influence each other although the underlying molecular mechanisms of this cross-communication remains largely unknown (238–241). Although the Hippo-mTORC1 crosstalk in pancreatic β-cells is just beginning to be understood, there is more and more new evidence linking Hippo

pathway as a regulator of mTORC1 and autophagy (and vice versa) in different cell types (242,243).

Hippo as regulator of mTOR. When there is a need for organ growth, the Hippo pathway will stay inactive, resulting in YAP/TAZ activation and specific pattern of gene expression to direct the processes towards cell proliferation and organ growth. There are several studies which show Hippo signaling components acting as direct regulators of mTOR and its associated signaling. Main downstream Hippo pathway effectors YAP/TAZ are strongly linked to the regulation of mTOR pathway. As reported in an elegant study, YAP/TAZ induces the expression of miR-29 which represses the expression of the tumor suppressor PTEN, an antagonist of PI3K, resulting in the activation of mTOR and cell growth in an epithelial cell line (240,244). Additionally, a recent study also suggests that YAP/TAZ affects amino acid induced mTORC1 activation by direct transcriptional regulation of amino acid transporter LAT1 (L-type amino acid transporter) which consists of heterodimeric complex of SLC7A5 and SLC3A2 (245). Namely, YAP/TAZ regulate mTORC1 activation by inducing transcription of SLC7A5 and increase amino acid uptake and subsequent amino acid mediated mTORC1 activation. Additionally, RASSF1A, well-known tumor suppressor and member of the Hippo pathway, can interact and form a complex with mTOR signaling component Rheb (246). Nelson and Clark demonstrated that RASSF1A-Rheb complex may potentiate the activation of the Hippo pathway but at the same time prevent the activation of mTORC1 by disrupting Rheb in human lung cancer cell lines. A recent study also suggests that YAP promotes mTOR expression and stability through the S-phase kinase-associated protein 2 (SKP2)-dependent manner in bladder cancer cells (247). SKP2 is an E3 ubiquitin ligase that belongs to the ubiquitin proteasome system and acts as an oncoprotein in multiple human cancers. YAP interacts with SKP2 to prevent mTOR ubiquitination thus increasing its stability and activity. Additionally, Gan *et al.* reported that LATS1/2 kinases directly phosphorylate Raptor at S606 and therefore inhibit mTORC1 activation in kidney HEK293 cells and MCF-7 breast cancer cells (241). LATS1/2 specifically phosphorylate Raptor and disrupt the interaction between Raptor and Rheb to enhance its binding to DEPTOR and suppress mTORC1 activity.

Hippo kinase MST1 also contributes to the regulation of mTORC1-AKT signaling. It physically binds to AKT in human prostate cancer (248) and glioma cells (249) and negatively regulates AKT-mTOR activity. Full-length MST1 as well as MST1 caspase cleavage products (N-terminal and C-terminal regions of MST1) function as AKT inhibitors in the cytoplasm and nucleus by directly interacting with AKT. As AKT regulation by phosphorylation is a more clearly established mechanism, the MST1 interaction potentially provides a negative feedback loop when AKT-mTORC1 is hyperactivated in tumors. So far, it was believed that autophagy

is mainly controlled through mTORC1 at upstream level but the signaling network might be much more complex than that. For instance, MST1/2 kinases phosphorylate the autophagosome membrane protein LC3 at T50 in mammalian mouse embryonic fibroblasts (MEFs), which is essential for mediating fusion of autophagosomes with lysosomes and efficient cargo degradation and clearance (250). Moreover, STK38 (the serine/threonine protein kinase 38; NDR1) is an important additional kinase in Hippo pathway controlled by MST1/2, which is able to phosphorylate YAP/TAZ leading to its proteasomal degradation (251,252). STK38 is also a positive regulator of autophagy that binds to Beclin1 forming Beclin1/STK38 complex and promotes early autophagosome formation opposite to MST1 (253,254).

mTOR as a regulator of Hippo. While through above discussed examples, the Hippo pathway regulates mTOR signaling. In turn, mTOR actively controls Hippo pathway and its related cellular processes: mTORC2 activates Hippo terminal effector YAP by two different mechanisms. In an indirect way, Collak *et al.* identified mTORC2 as direct negative regulator of Hippo pathway associated protein AMOTL2, leading to increased YAP activity in glioblastoma cells and in primary glioblastoma samples (255). Mechanistically, mTORC2-mediated phosphorylation of AMOTL2 at S760 prevents its interaction to YAP resulting in elevated nuclear translocation of YAP and activation of YAP target genes. AMOTL2 also acts as scaffold protein for LATS1/2 kinases (256) to promote LATS-mediated suppression of YAP. Such regulation of YAP is also lost due to mTORC2 action. In a direct way, Holmes *et al.* showed that mTORC2 directly phosphorylates YAP/TAZ at S436 and enhances its transcriptional activity (257). Specifically, mTORC2 mediated YAP phosphorylation promotes YAP stabilization, increases its nuclear localization and its transcriptional activity in a glioblastoma cell line. mTORC2 target AKT has also been previously shown to phosphorylate YAP at S127 in response to cellular damage (258). In addition, mTORC2 is also able to phosphorylate Hippo kinase MST1 at T120 and limits its ability to restrict cell growth and survival in prostate cancer cells and in tumor cells in vivo (259). Recent studies suggest that YAP/TAZ may also be under the control of AMPK, a key mTORC1 suppressor. Specifically, energy stress induced by glucose deprivation governs AMPK to directly phosphorylate YAP/TAZ at S94 and inhibits its transcriptional activity (260–262). Besides, AMPK also directly phosphorylates tight-junction protein AMOTL1 at S793. This stabilizes and increases AMOTL1 protein levels, which binds LATS1/2 and promote its kinase activity, leading to YAP/TAZ inhibition in HEK293A cells (263).

Altogether, these studies suggest that a dynamic interplay between Hippo and mTOR pathways can regulate cellular metabolism and growth in different cellular context.

1.5.1 mTOR-Hippo Pathway Crosstalk in Pancreatic β-cells

As mentioned, we identified LATS2 as an important direct upstream regulator of the mTORC1-autophagy signaling axis in stressed pancreatic islets (151). Specifically, LATS2 controls autophagy and defective autophagy-induced β-cell apoptosis by targeting mTORC1. Glucotoxic-associated conditions in pancreatic β-cells triggers LATS2 hyper-activation together with LATS2-mediated mTORC1 hyperactivation, ultimately leading to defective autophagy and β-cell apoptosis. Suppression of LATS2 results in reduced β-cell apoptosis and improved β-cell function in *in vitro* and *in vivo* experimental models of diabetes. We showed a novel cellular loop between LATS2, mTORC1 and autophagy, where LATS2 impairs autophagic flux through mTORC1 under chronic nutrient stress, but in turn autophagy itself can also directly control LATS2's lysosomal degradation to prevent aberrant LATS2 hyperactivity and accumulation during short, acute stress. Such regulatory loop is only one example of the tightly controlled stress-sensitive survival pathway in β-cells (151,264). Interestingly and contrary to our findings in the β-cell, Gan *et al.* show that LATS1/2 may directly phosphorylate Raptor, an essential component of mTORC1, at S606 to suppress the activation of mTORC1 (241) suggesting that a distinct regulation of mTORC1 by LATS1/2 may exist in different tissue or cell types. This is supported by the *in vivo* experiment using phosphomimetic (S606D; $Raptor^{D/D}$) knock-in mice, where LATS1/2 mediated Raptor-S606 phosphorylation is constitutively active. Whereas mTORC1 is active in the liver, heart and kidneys, its activation is reduced in the spleen and in the brain (241). We show that such regulation is not the case in β-cells. Overexpression of wild type (WT) Raptor as well as phospho-null (S606A) Raptor mutant similarly activates mTORC1 signaling in INS-1E β-cells. This suggests that phosphorylation Raptor-S606 is non-essential for mTORC1 activation in β-cells (151). Characterizing the Hippo-mTOR interplay in β-cells is only at the beginning and further investigation of their dynamic molecular crosstalk is needed.

1.6 PHLPP Phosphatases: Cellular Antagonist of Survival Pathways

Analyzing of human genome database for a novel phosphatase with PH domain, which can selectively dephosphorylate the hydrophobic motif of kinase AKT led to the identification of Pleckstrin homology (PH) domain leucine-rich repeat protein phosphatase 1 (PHLPP1) by Alexandra C. Newton and colleagues (265). PHLPP1 was initially discovered in the rat hypothalamus and named suprachiasmatic nucleus (SCN) of circadian oscillatory protein (SCOP) (266). SCOP protein was later renamed into PHLPP1, after its domain characterization (PH domain and Leucine rich repeat Protein Phosphatases). In 2007, the

same group (Newton et al.) discovered and characterized a second isoform of the PHLPP family, PHLPP2, and demonstrated that PHLPP2 could also dephosphorylate AKT (267). Thus, the PHLPP protein family consists of two isoforms, PHLPP1 and PHLPP2, with a similar domain structure. PHLPP1 is a larger isoform with 1717 amino acids, whereas PHLPP2 (also known as PHLPPL), consists of 1323 amino acids (Fig. 6). PHLPP1 and PHLPP2 are members of the protein phosphatase 2C (PP2C) family of serine/threonine phosphatases and together with others they form a metal-dependent protein phosphatase (PPM) family requiring magnesium or manganese for their activation (268).

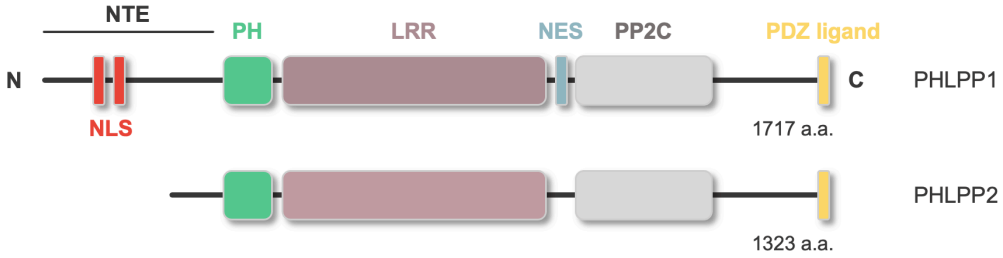

Figure 6: Domain architecture of PHLPP1/2. PHLPP1/2 are PP2C phosphatases belonging to a metal-dependent protein phosphatase (PPM) family. The structural unit of PHLPPs contain: N-terminal extension (NTE) containing a bipartite nuclear localization signal (NLS), a pleckstrin homology (PH) domain, a series of leucine-rich repeats (LRR), a nuclear export signal (NES), a protein phosphatase domain (PP2C) and a PDZ binding motif toward the C terminal (adapted from (269)).

PHLPP phosphatases act mostly through dephosphorylation of residues on the hydrophobic motif of their substrates in the cytosol, nucleus and mitochondria. Gao *et al.* first showed that PHLPP acts as serine/threonine phosphatase that controls AKT activity (265). PHLPP is able to negatively regulate mTORC2's phosphorylation site S473 on AKT by direct dephosphorylation. This drastically reduces AKT activity and suppresses its pro-survival effect. After the identification of the initial PHLPP substrate, AKT, many others followed. For example, PHLPP directly interacts with pro-apoptotic MST1 and dephosphorylates inhibitory T387 site, which activates MST1 and its downstream effector JNK to induce apoptosis (270). Gao *et al.* further demonstrated that PHLPP1/2 also suppress a key regulator of cell cycle progression and differentiation, PKC. PHLPP1/2 promote PKC downregulation by dephosphorylating its hydrophobic motif at S660, which is necessary in the resistance to degradation (271). Another study characterized Raf1 as a next important PHLPP substrate (272). They showed that PHLPP negatively regulates MAPK signaling implemented in cell growth by directly dephosphorylating Raf1 as S338, which inhibits its kinase activity. PHLPP can also suppress protein translation initiation and thus cell growth via direct

dephosphorylation of S6K1 at T389 (mTORC1 phosphorylation site) (273). One of the most recently identified substrates of PHLPP is the transcription factor signal transducer and activator of transcription 1 (STAT1) which is responsible for the transcription of inflammatory genes and regulation of inflammatory signaling. PHLPP1 decreases STAT1 transcriptional activity in macrophages by direct dephosphorylation of S727 and is therefore directly involved in suppression of the innate immunity and cytokine signaling (274). Over these years PHLPPs have captured the attention due to their ability to regulate crucial pathways affecting cell proliferation, growth, and apoptosis. In particular, its dysregulation can lead to serious pathophysiological conditions such as cancer, diabetes or cardiovascular diseases (275–279).

In principle PHLPPs are strong tumor-suppressor phosphatases with pro-apoptotic and anti-proliferative functions. PHLPP repress proliferative and survival pathways mostly through dephosphorylation and inactivation of pro-survival protein such as AKT, S6K, RAF1 and PKC. PHLPPs also decrease the expression of many receptor tyrosine kinases (RTKs), including insulin receptor (INSR), the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (280). For example, PHLPP1/2 dephosphorylate the transcription factor c-Jun (part of the activator protein-1 complex (AP-1)) at S63/73 and suppress its transcriptional activity leading to reduced EGFR levels (281,282). Thus, PHLPP-mediated suppression of MAPK signaling involves both, a direct inactivation of pathway components through dephosphorylation of Raf1, as well as indirect inactivation of transcription factor c-Jun which ultimately downregulate expression of EGFR. To enhance the apoptotic pathway and cell death, PHLPP dephosphorylates the pro-apoptotic kinase, MST1, via removal of an inhibitory phosphorylation as mentioned above (Fig. 8) (269).

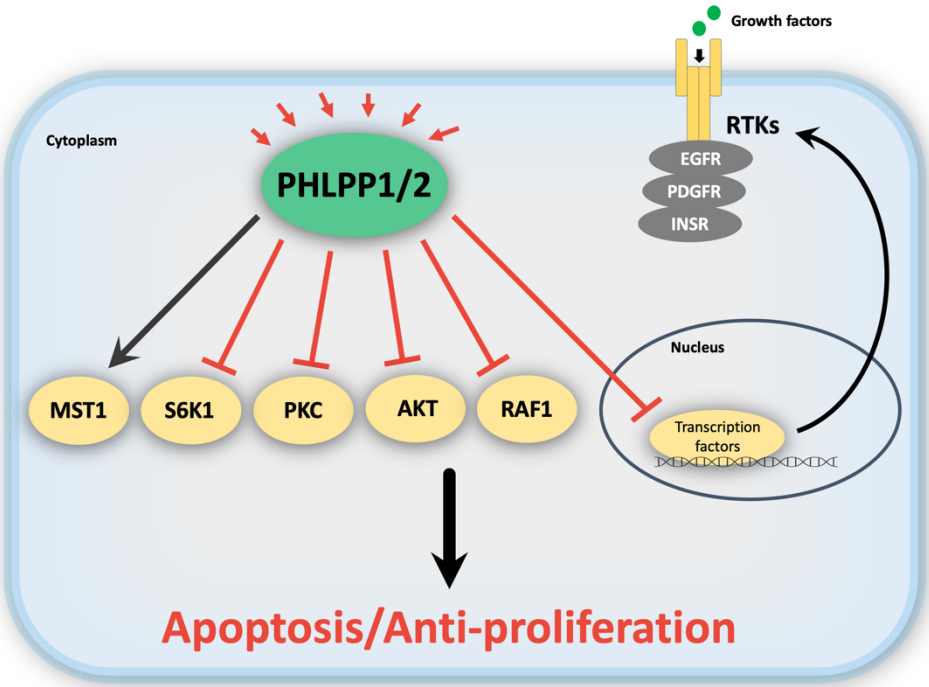

Figure 8: PHLLP as a pro-apoptotic and anti-proliferative mediator. PHLPP suppresses cell proliferation and survival through direct dephosphorylation and suppression of AKT, S6K1 and RAF1, and indirectly by inhibiting the transcription of many RTKs (e.g., INSR, EGFR and PDGFR) through the dephosphorylation of respective transcription factors. It also inactivates PKC and thus inhibit cell cycle progression and differentiation. Additionally, it promotes pro-apoptotic kinase, MST1, through removal of an inhibitory phosphorylation and contribute to apoptotic pathway initiation. PKC, protein kinase C; AKT/PKB, protein kinase B; IRSR, insulin receptor substrate receptor; EGFR; epidermal growth factor receptor PDGFR, platelet-derived growth factor receptor; INSR, insulin receptor; RTK; receptor tyrosine kinase; S6K1, ribosomal S6 kinase 1, PHLPP, pleckstrin homology domain leucine-rich repeat protein phosphatase (adapted from (269)).

PHLPP as a tumor suppressor. Due to its high pro-apoptotic effect, PHLPP has been more extensively studied in many cancer types, where PHLPP levels are largely reduced or lost (reviewed in (283)). Briefly, in glioblastoma cell lines, PHLPP1 gene expression is 40% lower compared to control (68). Similar is seen in chronic lymphocytic leukemia where PHLPP1 mRNA is down in 50% of cases (284). In melanoma, in which the AKT pathway is activated in 70% of cases, PHLPP1 mRNA and protein levels are reduced indicating an inverse correlation between PHLPP and AKT (285). In colon cancer, the expression of PHLPP1/2 levels is also decreased (286). Dysregulation of PHLPP in tumors may occur through various regulatory mechanism such as PHLPP-associated protein scaffolds and/or (post)-transcriptional and (post)-translational modifications of PHLPPs (extensively reviewed in (269)). For example, in glioblastoma tumors PHLPP1 is frequently defective due to downregulated NHERF1 scaffold

protein, which localize PHLPP to the plasma membrane for suppression of AKT (287). Likewise, the scaffold protein of the ligase complex, Cullin4B (CUL4B), can also inhibit PHLPP1/2 expression by binding their promotor region (288). Moreover, in melanoma AKT is hyperactivated due to hypermethylation of PHLPP1 gene, leading to its transcriptional suppression (285). In contrast, high levels of PHLPP1 mRNA in cartilage from patients with osteoarthritis correlated with reduced PHLPP1 promoter methylation and low proliferation due to lack of AKT activation (289). Similarly, transcriptional induction of PHLPP1 is also deregulated by binding of HDAC3 to PHLPP1 promotor region, resulting in decreased PHLPP1 level and AKT activation in chondrocyte hypertrophy (290). Liu *et al*, identified both PHLPPs as a translational target of mTORC1, which control the translation of PHLPP mRNA in human colon cancer cells (291). Due to high activation of AKT-mTORC1 axis in many cancer types, PHLPPs might be a component of compensatory feedback loop in which the activation of AKT is inhibited by up-regulation of PHLPPs through mTORC1. Additionally, PHLPPs mRNA is post-transcriptionally regulated by numerous miRNAs (e.g., miR-224, miR-15a and miR-375), resulting in decreased PHLPP protein expression and, consequently, enhanced AKT activation (292,293).

Due to their high contribution of AKT and MST1 to the regulation of cell homeostasis and vital survival pathways, these kinases should be carefully modulated to maintain healthy cells. Thus, PHLPPs and their two important targets AKT, and MST1 constitute an autoinhibitory triangle that controls the fine balance between apoptosis and proliferation (270).

PHLPPs' Role in Diabetes. Since PHLPPs are negative regulators of key survival pathways they are mostly studied in the context of cancer. Nevertheless, deregulated PHLPP is also associated with metabolic disorders such as obesity and diabetes (294,295). For example, a recent study showed that PHLPP2 is degraded in obesity-induced fatty liver. Potassium channel tetramerization domain containing 17 (KCTD17) is upregulated in liver tissues of HFD- or db/db obese mice and patients with non-alcoholic fatty liver disease (NAFLD). KCTD17 binds PHLPP2 to promote its ubiquitin-mediated degradation, which increases expression of genes that regulate lipogenesis, increasing lipid accumulation and hepatic steatosis (296). Likewise, PHLPP2 expression in hepatocyte plays a protective role by terminating insulin-induced AKT signaling to prevent excessive haptic lipid accumulation (297). In contrast, Kim *et al.* reported that elevated adipocyte PHLPP2 causes diet-induced obesity and fatty liver. While HFD-induced obese mice showed increased adipocyte accumulation, adipocyte specific deletion of PHLPP2 prevent adipocyte hypertrophy due to increased lipolysis. PHLPP2 deficiency led to increased PPARα-dependent adiponectin secretion, which in turn increases hepatic fatty acid oxidation to ameliorate obesity-induced

fatty liver (298). Moreover, elevated PHLPP1 expression is associated with impaired insulin action in adipose and skeletal muscle tissues of obese patients and patients with T2D (275) as well as in the liver of insulin-resistant rodents (299). PHLPP1 single-nucleotide polymorphism (SNP) has also been linked to T2D, indicating the importance of PHLPP1 in diabetes progression (300). Hribal *et al.* further showed that PHLPP1/2 are upregulated in the INS-1E β-cell line chronically exposed to high glucose concentrations and that reduction in PHLPPs expression restores a pro-survival profile (301).

Pancreatic islets have a very fragile microenvironment which is highly sensitive to different external and internal stressors. AKT largely contributes to the regulation of β-cell homeostasis to keep a healthy pool of β-cells. Recent observations showed that the chronic high glucose exposure is accompanied by an increased PHLPPs expression and reduction of the phosphorylation levels of AKT (302). As reported, activation of AKT and inhibition of GSK3 in β-cells is beneficial to promote PDX1 activity and delay its degradation (303). As mentioned before, MST1 is a strong mediator of apoptosis in diabetic islets and phosphorylates PDX1, leading to its proteasomal degradation. Consistent with these molecular mechanisms, potential upregulation of PHLPP levels in diabetic islets/ β-cells might therefore enhance deactivation of AKT as well as activation of MST1, leading to increased PDX1 degradation with a negative effect on β-cell survival and function. Thus, PHLPPs might serve as a novel upstream regulator of β-cell survival and insulin secretion. However, PHLPP expression and its function as well as their underlying mechanisms have not been explored and characterized in β-cells *in vitro* and *in vivo* and require further detailed investigation.

1.7 Aims of my thesis

Loss of functional pancreatic β-cell mass is the common pathogenic hallmark in all forms of diabetes due to increased β-cell apoptosis and/or β-cell dedifferentiation. Identification of key regulatory mechanisms and active signaling pathways involved in β-cell failure in diabetes is crucial to further complete the current knowledge of disease pathogenesis and is necessary for future therapeutic strategies aiming to prevent β-cell failure or repair dysfunctional β-cell in diabetes. As described above Hippo signaling is the key intracellular pathway, responsible for the regulation of cell proliferation and apoptosis. Several lines of evidence establish the importance of the critical Hippo pathway component, MST1, as a main regulator of apoptosis in diabetic pancreatic β-cells. Also, our molecular exploration and mechanistic investigation support that MST1 upstream regulators PHLPP1/2 phosphatases are highly upregulated in the diabetic islets/ β-cells and can promote β-cell dysfunction and death. In this dissertation, I put my major efforts to identify molecular events and cellular mechanisms that are responsible for β-cell failure in diabetes.

The first part and aim of my thesis was:

- to prove efficacy of MST1 inhibitor neratinib to prevent β-cell apoptosis and restore β-cell function and survival in several experimental models of diabetes in *in vitro, ex vivo* and *in vivo* settings.

In the second part I aimed to identify the detailed actions of PHLPPs in β-cells, namely whether:

- PHLPP levels are elevated in metabolically stressed human and rodent diabetic β-cells.
- PHLPPs' up-regulation leads to β-cell apoptosis and impaired insulin secretion in diabetes.
- PHLPPs directly dephosphorylate and regulate activities of their downstream targets, kinases AKT and MST1.
- diabetes associated hyper-activation of mTORC1 is the primary mechanism of PHLPPs' upregulation in stressed β-cell.
- genetic inhibition of PHLPPs improves β-cell survival and function in experimental models of diabetes in vitro, in vivo and in primary human T2D islets.

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2 Publications

Publication I

Neratinib protects pancreatic beta cells in diabetes

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My contribution:
Designed, performed experiments, analysed data and assembled the figures for: Figures 2; 3 A,B; 4 C; 5 B,C; 9 C,D
Supplementary Figure 4

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Neratinib protects pancreatic beta cells in diabetes

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The loss of functional insulin-producing β-cells is a hallmark of diabetes. Mammalian sterile 20-like kinase 1 (MST1) is a key regulator of pancreatic β-cell death and dysfunction; its deficiency restores functional β-cells and normoglycemia. The identification of MST1 inhibitors represents a promising approach for a β-cell-protective diabetes therapy. Here, we identify neratinib, an FDA-approved drug targeting HER2/EGFR dual kinases, as a potent MST1 inhibitor, which improves β-cell survival under multiple diabetogenic conditions in human islets and INS-1E cells. In a pre-clinical study, neratinib attenuates hyperglycemia and improves β-cell function, survival and β-cell mass in type 1 (streptozotocin) and type 2 (obese $Lepr^{db/db}$) diabetic mouse models. In summary, neratinib is a previously unrecognized inhibitor of MST1 and represents a potential β-cell-protective drug with proof-of-concept in vitro in human islets and in vivo in rodent models of both type 1 and type 2 diabetes.

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Loss of function and/or mass of pancreatic β-cells is a critical pathogenic hallmark of both type 1 and 2 diabetes (T1D/T2D)[1–5]. Pancreatic β-cell apoptosis contributes to the loss of insulin-producing β-cells in diabetes, rapidly induced by the activation of the immune system in T1D and slowly progressing in T2D[1–4,6–11]. In addition, β-cell dedifferentiation[12–14] and failure of adaptive expansion due to impaired proliferation[15,16] are other proposed mechanisms for the loss of functional β-cell mass in diabetes. The mechanisms of β-cell failure are complex; multiple triggering factors have been identified, which initiate signaling cascades that affect the expression of apoptotic genes. The development of novel agents that can selectively block β-cell apoptosis together with the restoration of β-cell function with safety profiles commensurate with the treatment of chronic disease is urgently needed. Current therapies for the treatment of diabetes are directed toward alleviating only the symptoms, i.e., the normalization of glycemia through enhanced insulin secretion from the remaining β-cells, and the improvement of insulin sensitivity in T2D, and through tightly controlled exogenous insulin therapy in T1D. None of the currently used antidiabetic agents target the maintenance of endogenous β-cell mass, although it has been demonstrated that even a small amount of preserved endogenous insulin secretory function has great clinical benefits[17].

In our previous work, we identified mammalian sterile 20-like kinase 1 (MST1, also known as STK4, KRS2) as a critical regulator of pancreatic β-cell death and dysfunction[11]. MST1 is a ubiquitously expressed serine/threonine kinase, the major upstream signaling kinase in the Hippo pathway, involved in multiple cellular processes, such as morphogenesis, proliferation, stress response, and apoptosis[18,19]. MST1 is a direct target as well as an activator of caspases, forming a feed-forward loop that drives the apoptotic signaling pathway[20,21]. MST1 promotes cell death through regulation of multiple downstream targets, such as LATS1/2, histone H2B, FOXO family members, the intrinsic mitochondrial proapoptotic pathway, stress kinase c-Jun-N-terminal kinase (JNK), and caspase-3 activation[19,22,23]. MST1 is strongly activated in β-cells under diabetogenic conditions and its activity correlates with β-cell apoptosis and degradation of PDX1[11,24], a β-cell transcription factor highly important for β-cell identity, survival, and function[25]. MST1 deficiency markedly restores β-cell function and survival and leads to protection of β-cell mass and normoglycemia in mouse models of diabetes[11]. The identification and elaboration of MST1 inhibitors represents a promising approach to β-cell-protective drugs for the treatment of diabetes.

Several series of MST1 inhibitors have been reported, demonstrating the feasibility of generating potent, selective small-molecule inhibitors[26–29]. Through a biochemical MST1 inhibition screen across a highly privileged collection of 641 drug-like kinase inhibitors, we identified neratinib as a potent MST1 inhibitor. Neratinib is a covalent, irreversible ATP-competitive dual inhibitor of HER2/EGFR. The epidermal growth factor receptor (EGFR, also named ErbB-1/HER1) and human epidermal growth factor receptor 2 (HER2, also named ErbB-2) are tyrosine kinases of the ErbB family and involved in organ development and growth, as well as in the pathogenesis of various tumors[30]. FDA approved for the treatment of breast cancer[31–33], neratinib is also in clinical trials for lung, colorectal, and bladder cancers. Via its acrylamide moiety, neratinib forms a covalent interaction with the conserved cysteine residue (Cys-773 in EGFR and Cys-805 in HER2), resulting in tight engagement of the ATP-binding site and robust inhibition of the activation of the EGFR signaling pathway and cell proliferation[34]. However, this conserved cysteine is not present in MST1.

In this study, we report neratinib as a β-cell-protective kinase inhibitor in proof-of-concept experiments in a widely used β-cell line, in human islets, as well as in both T1D and T2D rodent models. Specifically, the goal of this work was to evaluate neratinib's efficacy to prevent apoptosis in human islets and to restore normoglycemia in the streptozotocin (STZ)-induced and in the obese $Lepr^{db/db}$ diabetic mouse models.

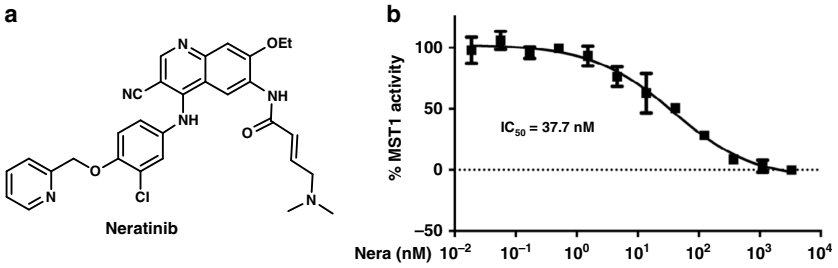
Results

Neratinib was identified as MST1 inhibitor. To identify novel MST1 inhibitors, we developed a high-throughput LanthaScreen Eu kinase binding assay platform in 1536-well format. A focused library of 641 annotated kinase inhibitor compounds was screened at 5 concentrations (1, 0.2, 0.04, 0.008, and 0.0016 μM). With staurosporine as the positive control, we chose hits with ≥75% inhibition of binding at 1 μM and ≥50% inhibition at 0.2 μM compared with staurosporine, which exhibited 100% inhibition at 1 μM. A total of 39 hits were selected for 9-point dose response confirmation in triplicate, starting at 1 μM followed by 1:5 serial dilution. Neratinib was identified as a potent inhibitor of MST1 (IC_{50} = 37.7 nM for the binding assay) (Fig. 1a, b).

We then re-profiled neratinib in a representative panel of 50 serine, threonine, and tyrosine kinases, which revealed inhibition of 16 serine/threonine and tyrosine kinases with >50% of inhibition at 10 μM, including 98% MST1 inhibition by neratinib (Fig. 1c). Following the above results, we expanded the kinase assay panel to 250 kinases, which revealed inhibition of 59 serine/threonine and tyrosine kinases with 50% of inhibition at 3 μM, reconfirming 97% MST1 inhibition by neratinib (IC_{50} = 91.4 nM for the activity-based assay; Supplementary Fig. 1a). We further evaluated these targets in dose–response experiments to 38 kinases in the panel, including EGFR, MAP4K4, MST1, and MST2, which showed consistent potency of neratinib on these four kinases, as well as potent inhibition on LOK, MAP4K5, and YES (Supplementary Fig. 1b). Further expanded dose–response experiments revealed neratinib's IC_{50} values of 1.79 nM for the well-known EGFR inhibition, 33.48 nM for MAP4K4, and 87.81 nM for MST2, indicating only a limited activity of neratinib toward MST2 (Supplementary Fig. 2).

Neratinib blocks MST1 activation and apoptosis in β-cells. To identify whether neratinib can inhibit MST1 activation and restore β-cell survival under chronic diabetogenic conditions, we exposed the INS-1E cells to various stress conditions in vitro (oxidative stress: H_2O_2, increasing glucose concentrations alone: glucotoxicity or in combination with palmitic acid: glucolipotoxicity, and ER stress: thapsigargin). As shown previously[11], MST1 was highly upregulated by all diabetic conditions upon chronic exposure, shown by its autophosphorylation (pMST1–T183; Fig. 2). In contrast, neratinib potently inhibited H_2O_2- and high glucose/palmitate-induced MST1 activation and apoptosis as represented by caspase-3 and PARP cleavage in β-cells (Fig. 2a–c). Also, neratinib restored PDX1 expression in β-cells, which was reduced by elevated glucose concentrations (Fig. 2c).

Caspase-3 activation induced by the ER stressor thapsigargin was dose-dependently abolished by neratinib, as determined by the NucView 488 caspase-3 assay (Supplementary Fig. 3a) confirming our previous data showing MST1 and caspase-3 activation by thapsigargin in β-cells, and the prevention of thapsigargin-induced apoptosis by caspase-3 inhibition[11]. Similarly, caspase-3 activation induced by the complex mixture of inflammatory cytokines (TNFα/IFNγ) and high glucose (33 mM; Supplementary Fig. 3b) as well as lipooligosaccharide (LPS)-induced expression of inflammatory cytokines TNFα, IL-1β, and IL-6 was largely inhibited by neratinib (Supplementary Fig. 3c). Neratinib treatment showed no evidence of interference on basal



c

Kinase	% inhibition by neratinib	Kinase	% inhibition by neratinib
ABL1	36	LCK	96
AMP-A1B1G1	11	MAP4K4	73
AURORA-A	28	MAPK1	12
CAMK4	19	MAPKAPK-2	7
CDK1	14	MEK1	73
CDK2	9	MEK6	9
CHEK1	27	MET	28
CK1-EPSILON	77	MST1	98
CSK	95	MST2	97
DAPK1	4	NEK2	68
DYRK1A	3	P38-ALPHA	15
EGFR-002	99	PAK2	101
EPH-A2	51	PDGFR-ALPHA	31
FAK	8	PDK1	6
FGFR1	26	PI3-KINASE-ALPHA	18
FLT-3	47	PIM-1-KINASE	13
GSK-3-BETA	23	PKC-ALPHA	7
IGF1R	37	PRKACA	3
IKK-BETA	−7	PRKD2	71
IRAK4	17	PYK2	41
ITK	88	ROCK1	−48
JAK1	5	SGK1	28
JAK2	18	SRC	73
JNK2	84	SYK	−14
KDR	64	TYK2	15

Color code
> 80%
50~79%
< 50%

Fig. 1 Neratinib, a kinase inhibitor with MST1 efficacy. **a** Chemical structure of neratinb. **b** Biochemical dose response confirmation of MST1 inhibition. Data show means ± SEM from three independent experiments ($n = 3$). **c** Kinase profiling showing % kinase inhibition at 10 μM neratinib with a panel of 50 kinases, means of duplicates are shown. Source data for (**b**) are provided as a Source Data file

cell viability as determined by steady-state ATP concentrations in INS-1E β-cells at all tested concentrations (Supplementary Fig. 3d).

Neratinib blocks MST1 activation and apoptosis in islets. The efficacy of neratinib to restore β-cell survival under multiple diabetogenic conditions was confirmed in six independent experiments by using human islet preparations from six different organ donors. Human islets were plated in a monolayer-like culture, and due to the complexity of the islet tissue culture, we also tested the higher concentration of 25 μM neratinib, which did not result in any detectable toxicity at basal control levels. Again, neratinib potently and significantly inhibited pro-inflammatory cytokine- as well as high glucose/palmitate-induced MST1 activation and caspase-3 activation in human islets (Fig. 3a, b). Further analysis of TUNEL/insulin co-positivity in isolated human (Fig. 3c, d) as well as in mouse islets (Fig. 4f, g) confirmed the anti-apoptotic action of neratinib indicating its β-cell-specific protective effect against diabetogenic condition-induced apoptosis in both primary human and mouse isolated islets.

Neratinib blocks MST1 signaling and β-cell apoptosis. Further analyses in INS-1E β-cells (Fig. 4a–c), human (Fig. 4d, e), and mouse islets (Fig. 4f, g) confirmed that the protective effect of neratinib on β-cell apoptosis was dependent on MST1. As we observed a parallel restoration of β-cell survival and MST1 inhibition, we aimed to identify whether neratinib can specifically interfere with MST1 downstream signaling and block MST1-induced apoptosis.

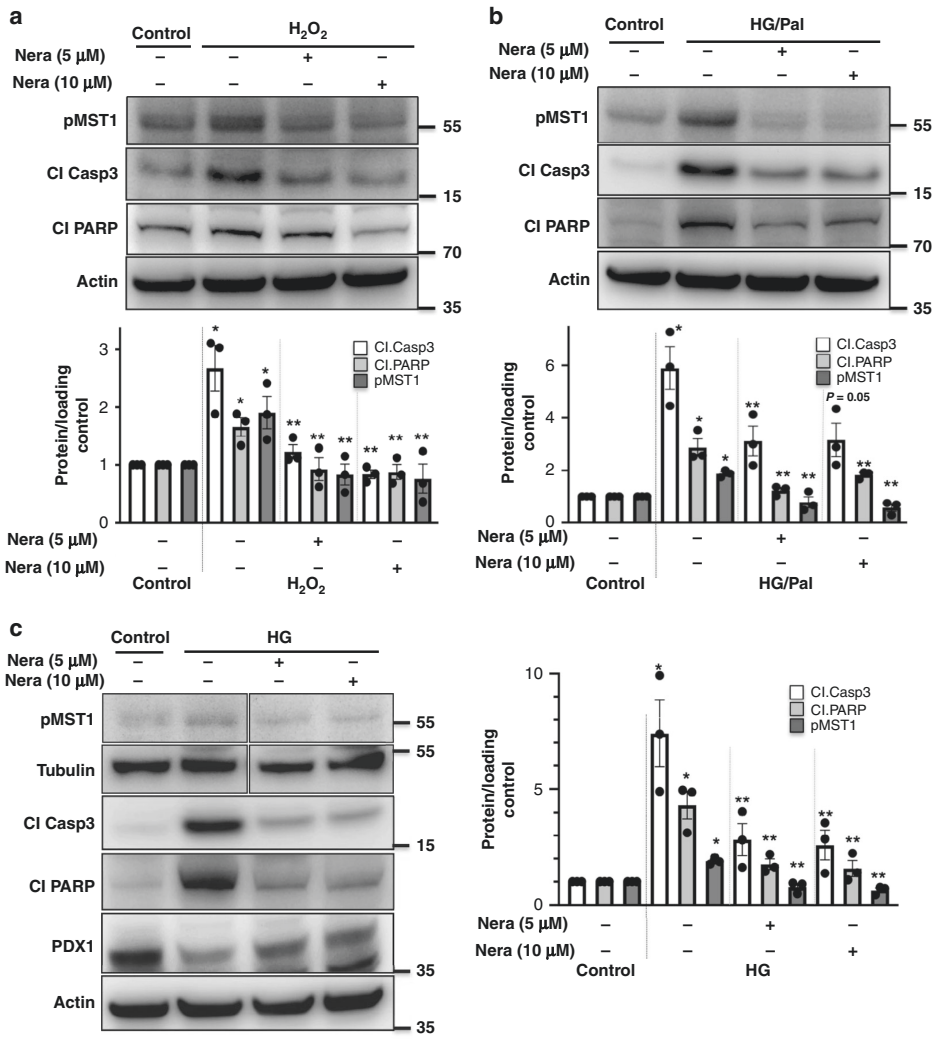


Fig. 2 Neratinib blocks MST1 activation and apoptosis in INS-1E β-cells. INS-1E cells were exposed to diabetogenic conditions (**a** H_2O_2, **b**, **c** 22.2 mM glucose or the mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Palm)) ± neratinib for 72 h. Phospho-MST1 (pMST1; pThr183), caspase-3 and PARP cleavage, PDX1, tubulin, and actin were analyzed by western blotting. Representative Western blots and pooled quantitative densitometry analysis are shown from three independent cell line experiments ($n = 3$). Results shown are means ± SEM. *$p < 0.05$ H_2O_2 or HG or HG/Pal to control, **$p < 0.05$ neratinib to vehicle-treated β-cells under the same diabetogenic conditions; all by Student's *t* tests. Source data are provided as a Source Data file

Recently, a highly sensitive and reproducible bioluminescence-based biosensor (LATS-BS) that monitors the specific activity of MST1 and its downstream substrate LATS kinase in vitro in real time was developed[35]. Both MST1 and LATS2 are core kinases of Hippo signaling pathway, which act together to induce β-cell apoptosis[36], and the specific MST1–LATS2 signaling activation can therefore be analyzed by this assay. LATS1/2 kinases phosphorylate their own established target Hippo transcriptional coactivator yes-associated protein (YAP) on S127 that exposes the docking site for binding of 14-3-3 proteins and leads to YAP cytoplasmic sequestration. Therefore a LATS-BS construct has been generated with fusion of YAP fragment and 14-3-3 with N-terminal and C-terminal firefly luciferase fragments (N-luc and C-luc), respectively that assesses LATS kinase activity by measuring the interaction between pS127-YAP and 14-3-3[35] in a MST1–LATS2-phosphorylation-dependent manner (Fig. 4a). Adenoviral overexpression of MST1/LATS2 in YAP-deficient INS-1E[37] β-cells transfected with LATS-BS induced strong bioluminescence-based induction of luciferase activity (represents YAP-14-3-3 final interaction; Fig. 4b) as well as YAP-S127 phosphorylation as determined by YAP-S127 phospho-specific antibody (Fig. 4c), both which was strongly inhibited by neratinib indicating its potent inhibitory action against MST1–LATS2 signaling, while canertinib, a related acrylamide-based covalent EGFR inhibitor with a similar structure to neratinib but without MST family inhibitory activity[38] added at the same conditions had no inhibitory effect.

Consistent with our previous observations that MST1 over-expression alone was sufficient to induce β-cell apoptosis[11], adenoviral overexpression of MST1 induced a dramatic induction of β-cell apoptosis in isolated human islets, which was significantly blocked by neratinib (Fig. 4d, e and Supplementary Fig. 4) suggesting a direct interference of neratinib with proapoptotic MST1 or its downstream signaling. To see whether

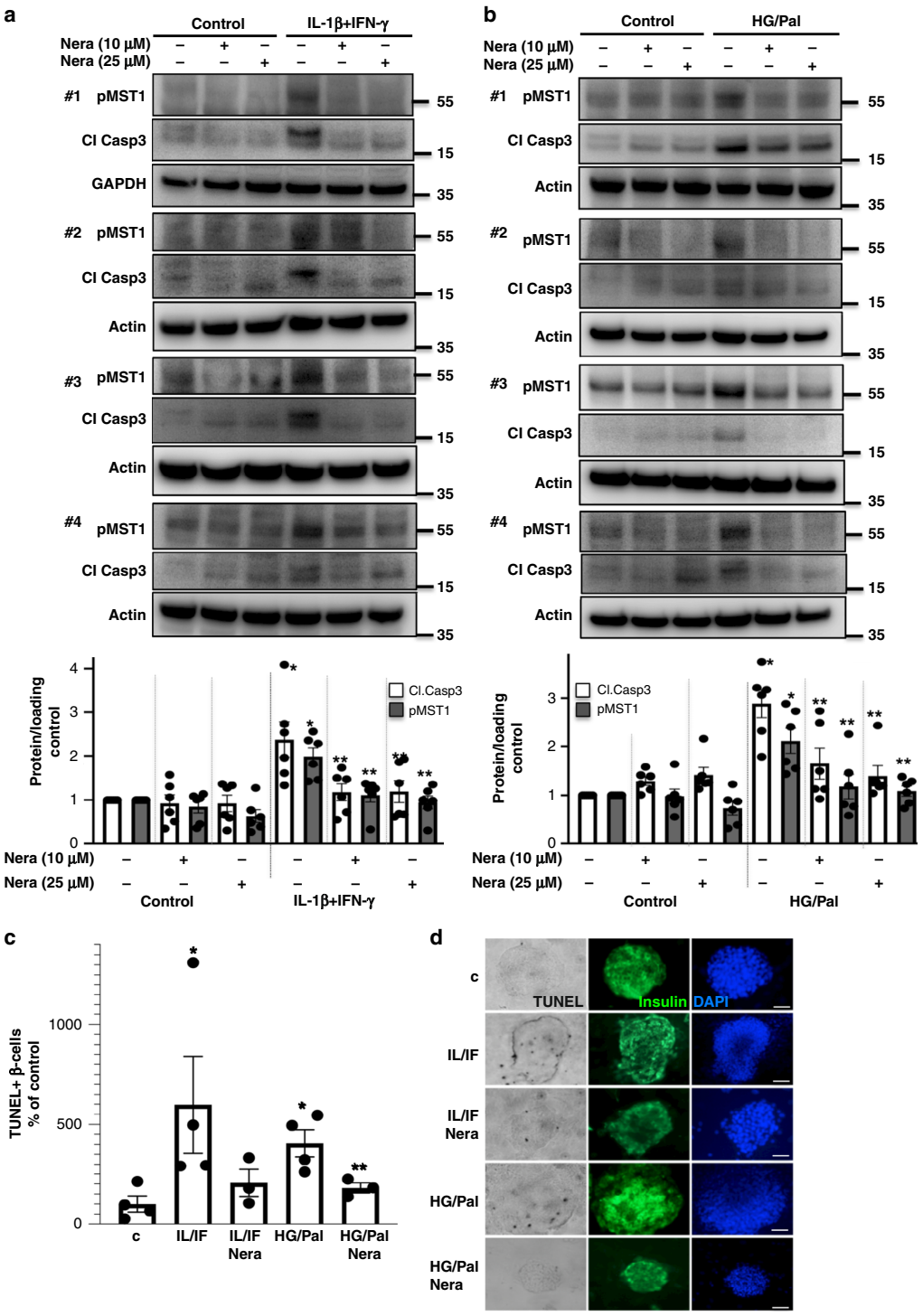
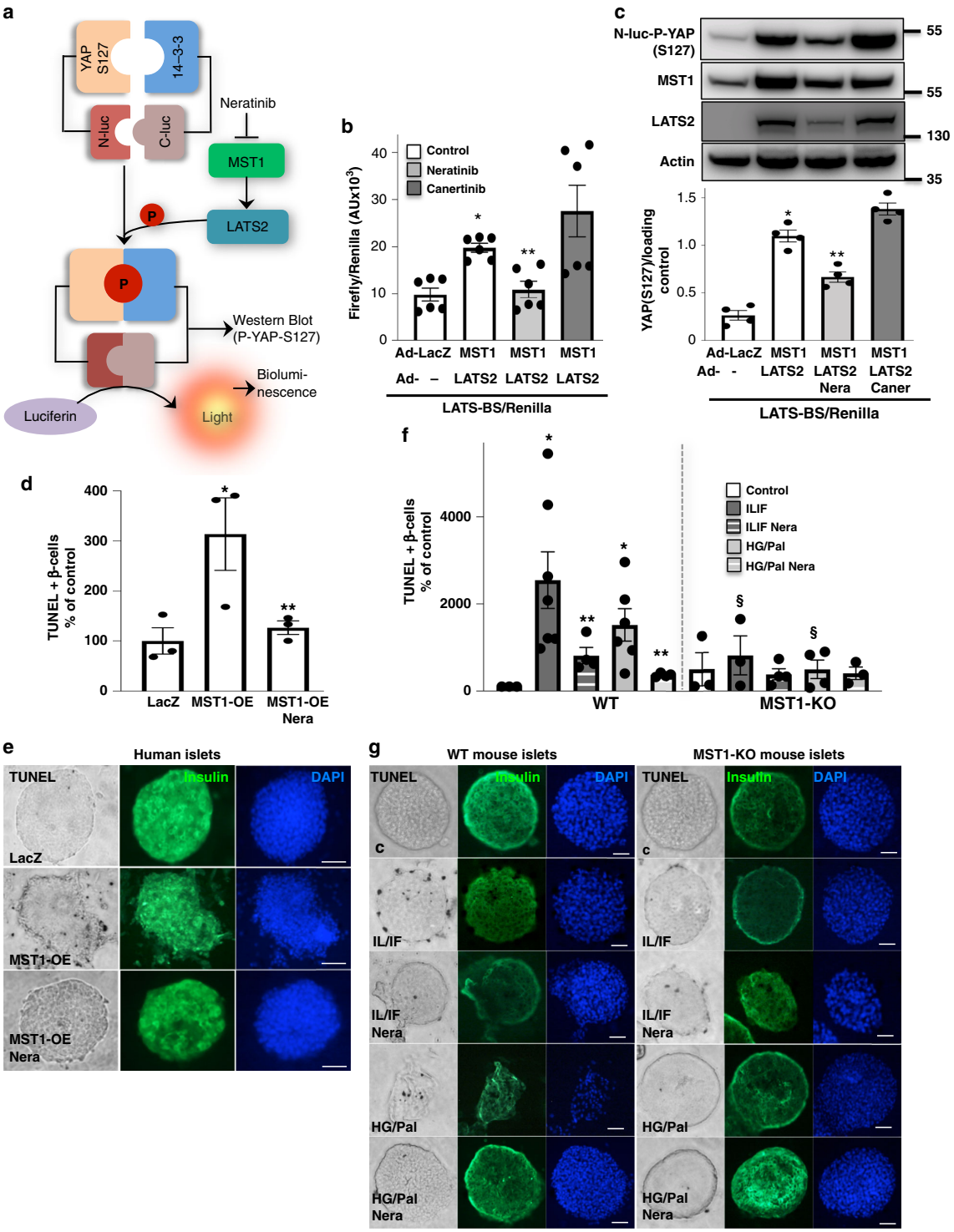


Fig. 3 Neratinib blocks MST1 activation and apoptosis in human islets. Human islets were exposed to diabetogenic conditions (**a**, **c**, **d** IL-1β/IFNγ, **b**–**d** mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Palm)) ± neratinib for 72 h. **a**, **b** Phospho-MST1 (pMST1; pThr183), caspase-3 cleavage, and GAPDH or actin were analyzed by western blotting. Representative Western blots of four different human islet donors (**a**, **b**; upper panels) and pooled quantitative densitometry analysis (**a**, **b**; lower panels) of six different human islet donors are shown ($n = 6$). **c**, **d** β-cell apoptosis analyzed by triple staining of TUNEL (black nuclei), insulin (green), and dapi (blue). Scale bar, 100 μm. An average number of 40,420 insulin-positive β-cell per condition was counted in 3–4 independent experiments from 3 to 4 different human islet donors ($n = 3$–4). Results shown are means ± SEM. $^{*}p < 0.05$ IL/IF or HG/Pal to control, $^{**}p < 0.05$ neratinib to vehicle-treated islets under the same diabetogenic conditions; all by Student's *t* tests. Source data are provided as a Source Data file



MST1 is the true target of neratinib in the context of β-cell protection from apoptosis, we evaluated its effect in isolated islets from global MST1-knockout (MST1-KO) mice and their wild-type (WT) littermates. While pro-inflammatory cytokines and glucolipotoxicity highly induced apoptosis in WT mice, their harmful effect on β-cell survival was almost gone in islets isolated from MST1-KO mice (Fig. 4f, g), consistent with our previous observation[11]. Similarly, neratinib reduced cytokine- and glucolipotoxicity-induced apoptosis in WT islets, but had no additive effect in MST1-KO mice, assuming that MST1 inhibition by neratinib is sufficient to restore β-cell survival. Neratinib had no significant effect in MST1-KO islets, neither on cytokine- nor

Fig. 4 Neratinib blocks MST1 signaling and MST1-induced β-cell apoptosis. **a** Domain structure and mechanism of action for the LATS-BS. At control condition, there is no interaction between YAP and 14-3-3 showing minimal bioluminescence activity for LATS-BS (N-luc-YAP15-S127 and C-luc-14-3-3)[35]. Upon LATS activation induced by MST1, LATS-dependent phosphorylation of YAP15-S127 (analyzed by Western blotting in (**c**)) leads to 14-3-3 binding, luciferase complementation, and high biosensor signal corresponding to higher LATS activity (analyzed by bioluminescence in (**b**)). **b**, **c** Adenoviral overexpression of MST1/LATS2 or LacZ (control) in INS-1E cells, which had been transfected with the firefly luciferase reporter plasmids N-luc-YAP15-S127 and C-luc-14-3-3 as well as pRL-Renilla luciferase vector control 24 h before 10 μM neratinib or canertinib was added for the last 24 h. Downstream YAP-S127 phosphorylation was determined by luciferase activity (normalized to the Renilla signal (**b**)). Western blotting for YAP-127 phospho-specific antibody (**c**); successful transfection was confirmed by LATS2 and MST1 analysis, and actin was used as housekeeping control. Data are means from six independent culture dishes ($n = 6$; **b**) or four independent cell line experiments ($n = 4$; **c**) ± SEM. Representative Western blot is shown. **d**, **e** Human islets were infected with Ad-LacZ (control) or Ad-MST1 adenoviruses and exposed to 10 μM neratinib for 48 h. **f**, **g** Isolated islets from MST1-KO mice and their WT littermates were recovered after isolation overnight and exposed to diabetogenic conditions (IL-1β/IFNγ or the mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Pal)) ± 10 μM neratinib for 72 h. **d**–**g** β-cell apoptosis was analyzed by triple staining of TUNEL (black nuclei), insulin (green), and dapi (blue). Scale bar, 100 μm. **d** An average number of 30,700 insulin-positive β-cell per condition was counted in three independent experiments from three different donors ($n = 3$) and **f** of 5942 insulin-positive β-cells per condition from three to seven mice/condition ($n = 3$–7). Results shown are means ± SEM. $^{*}p < 0.05$ MST-OE, or IL/IF or HG/Pal to Lac-Z or control, $^{**}p < 0.05$ neratinib treated with MST1-OE or IL/IF or HG/Pal-treated islets to vehicle-treated islets under the same conditions; $^{\S}p < 0.05$ MST1-KO to WT islets under the same diabetogenic conditions; all by Student's *t* tests. Source data are provided as a Source Data file

in glucolipotoxicity-induced apoptosis; these results show that neratinib specifically blocks MST1 signaling and MST1-mediated β-cell apoptosis in islets under diabetogenic conditions.

Neratinib but not canertinib restores β-cell survival. To provide further incisive target validation data for MST1, cellular target engagement as well as functional studies were performed by using neratinib and a closely related EGFR inhibitor canertinib (a.k.a. CI-1033) that lacks MST1 inhibitory activity as a target control. Indeed, canertinib showed potent EGFR inhibition (IC_{50} value of 0.21 nM), but no appreciable inhibition of MST1 or MST2 at concentrations up to 10 μM in our biochemical kinase inhibition assays (Supplementary Fig. 5). Cellular target engagement assays such as the cellular thermal shift assay (CETSA) determine direct interactions between a drug and its protein target, based on drug- or ligand-induced thermal stabilization of target proteins in intact cells[39]. In this case, CETSA has been applied to assess the direct interaction of neratinib or canertinib with MST1 in INS1-E cells. Consistent with their biochemical kinase inhibition profile, a specific binding of neratinib to MST1 in live cells is suggested by MST1 stabilization at 55 °C, while its degradation occurred at the same condition with canertinib or vehicle control (Fig. 5a).

We then compared the effect of neratinib and canertinib on MST1 activation, its downstream apoptotic target, and β-cell apoptosis in metabolically stressed β-cells. While neratinib, consistently with presented data (Figs. 2 and 3), strongly counteracted stress-induced MST1 activation and caspase-3 and PARP cleavage, the EGFR inhibitor canertinib was ineffective at a similar concentration (Fig. 5b, c). We have previously shown that MST1 activates the mitochondrial pathway of cell death in β-cells through regulating the BH3-only protein member BIM of Bcl-2 family proteins and that MST1-induced apoptosis requires BIM to trigger mitochondrial-mediated apoptosis in β-cells[11]. Correspondingly, our data show that the EGFR/MST1 inhibitor neratinib but not the EGFR inhibitor canertinib significantly reduced downstream mitochondrial BIM induction under diabetic conditions (Fig. 5b, c). Unlike neratinib, canertinib showed a significant stimulatory effect on apoptotic effectors cleaved caspase-3, cleaved PARP, and BIM under basal conditions (Fig. 5b, c). These data suggest that EGFR signaling inhibition (as represented by canertinib) is dispensable for neratinib-induced MST1 inhibition and protection from apoptosis.

Neratinib restores glycemia in a T1D mouse model. Initial pharmacokinetic studies in mice (Supplementary Fig. 6a) were performed to determine the exposure and half-life of neratinib. Mice were food-deprived overnight, and neratinib (5 mg/kg in 30% PEG400/0.5% Tween80/5% propylene glycol in saline; single i.p. dose) was given to the mice, and plasma samples were collected 30 min., 1, 3, and 7 h post dosing. Neratinib displayed a stable exposure profile in mice plasma and reached the maximum concentration at 306 ng/mL, on average 0.67 h post dosing (Supplementary Fig. 6a).

Multiple low-dose streptozotocin (MLD–STZ) injections induce severe diabetes through activation of cell-intrinsic apoptotic pathways as well as selective immune-mediated destruction of β-cells. Since neratinib blocked apoptosis in human islets and in β-cells, we tested its ability to restore normoglycemia in vivo in a mouse model of MLD–STZ-induced β-cell demise and T1D. Neratinib treatment had no influence on body weight (Supplementary Fig. 6b). By day 3 of post-STZ treatment, hyperglycemia was evident, with glucose levels progressively increasing throughout the 35-day study (Fig. 6a). This was accompanied with severely impaired glucose tolerance in the STZ-treated control mice (Fig. 6b). Neratinib-treated mice had lower glucose levels during the entire 35 days of the study and exhibited significantly improved glucose tolerance. Neratinib had no effect on glycemia in nondiabetic control mice (Fig. 6a, b).

Insulin tolerance tests demonstrated dramatically elevated glucose levels during a 4-h fast prior to insulin administration (Fig. 6c), but neither neratinib nor STZ had a significant effect on insulin sensitivity, shown by the analysis of normalized glucose levels (Fig. 6d). Indeed, impaired insulin secretion observed in STZ-treated mice during an intraperitoneal glucose-stimulated insulin secretion assay was significantly improved with neratinib treatment (Fig. 6e). Consistently, insulin-to-glucose ratios were significantly elevated in neratinib-treated STZ mice (Fig. 6f). These data suggest that glucoregulatory effects of neratinib are primarily mediated by improved β-cell function. Furthermore, islet architecture was disrupted, leading to significantly reduced β-cell mass, in STZ-treated mice compared with nondiabetic control mice (Fig. 6g), as a result of profound increase in β-cell apoptosis (Fig. 6h and Supplementary Fig. 7). Together with the increased β-cell apoptosis induced by STZ, β-cell proliferation was also induced, indicative of compensatory capacity in response to STZ-induced β-cell injury (Fig. 6i and as reported before[11]). Neratinib restored β-cell mass and reduced β-cell apoptosis (Fig. 6g, h), with no effect on β-cell proliferation in either control or diabetic mice (Fig. 6i).

Neratinib restores PDX1, NKX6.1, and Glut2 expression. We next examined whether neratinib can also restore expression of

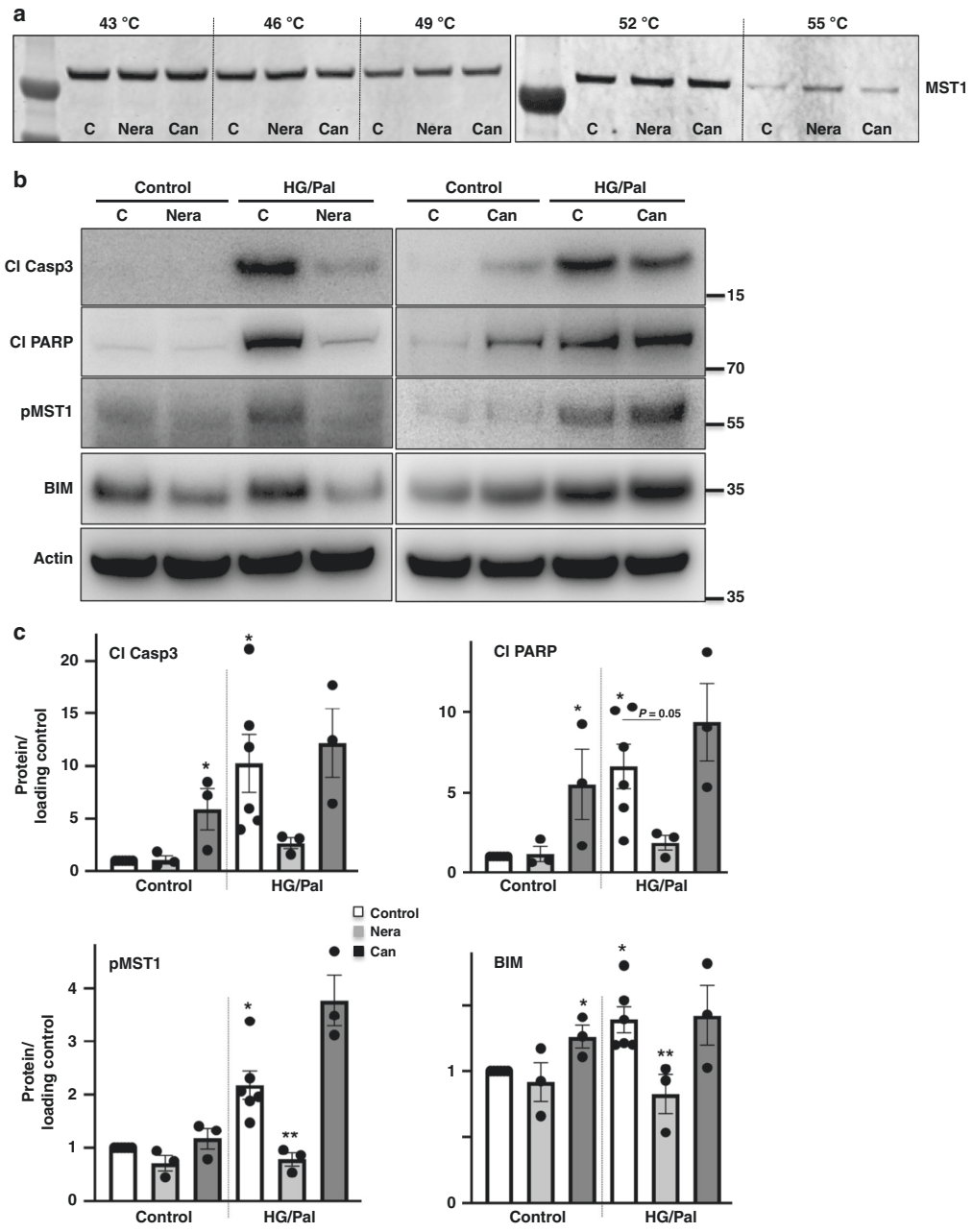
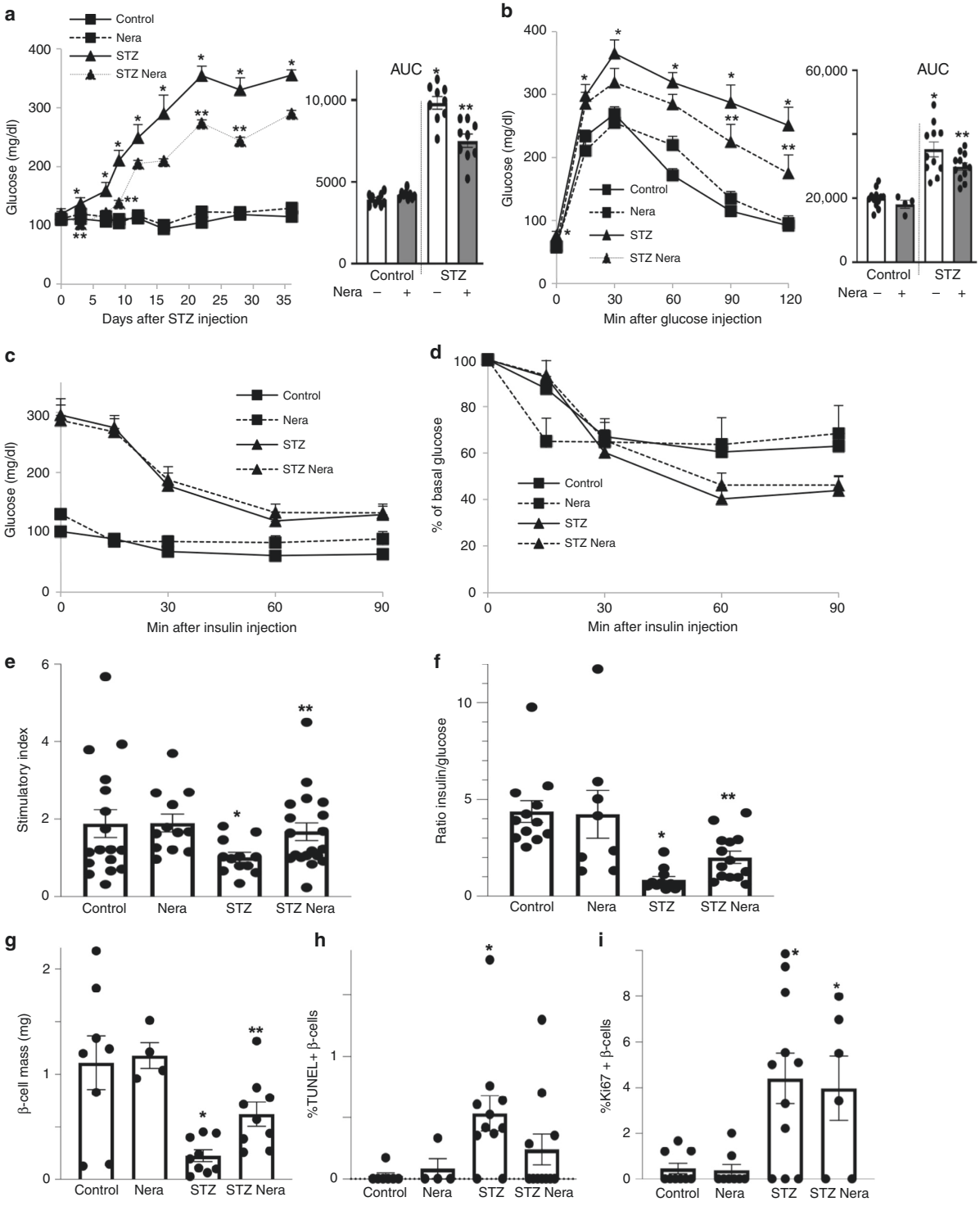


Fig. 5 Neratinib but not canertinib binds to MST1 and restores β-cell survival through MST1 inhibition. **a** CETSA assay was performed in INS-1E cells pretreated with 5 μM neratinib, canertinib, or vehicle control, followed by heating to denature and precipitate protein at different temperatures. The stabilized MST1 protein in the soluble fraction of the cell lysate was detected by using western blotting. **b**, **c** INS1-E cells were exposed to diabetogenic conditions (22.2 mM glucose and 0.5 mM palmitate (HG/Pal)) ± 10 μM neratinib or canertinib for 24 h. Phospho-MST1 (pMST1; pThr183), caspase-3 and PARP cleavage, and BIM and actin were analyzed by western blotting. Representative Western blots (**b**) and pooled quantitative densitometry analysis (**c**) are shown from 3–6 independent culture dishes ($n = 3$–6). Results shown are means ± SEM. *$p < 0.05$ HG/Pal to control, **$p < 0.05$ neratinib to vehicle + HG/Pal-treated β-cells under the same conditions; all by Student's t tests. Source data are provided as a Source Data file

three important markers for β-cell glucose metabolism and insulin production—the transcription factors PDX1 and NKX6.1 and the glucose transporter Glut2. MLD–STZ-treated mice showed impairment in the islet architecture with smaller islets and many insulin-negative cells together with reduced numbers and expression of nuclear PDX1- and NKX6.1-positive cells. Many cells within the islets, which still express insulin, had lost their NKX6.1 expression. These effects were markedly attenuated by neratinib treatment (Fig. 7a, b). The PDX1 target gene Glut2 was largely preserved in β-cell membranes of control mice, while the disrupted islet architecture of diabetic mice was also apparent by Glut2 staining, which was barely detectable in the MLD–STZ-



treated mice (Fig. 7c). Neratinib treatment restored β-cell Glut2 expression (Fig. 7c). These effects of neratinib confirm previous results from MST1-KO mice, where PDX1 and Glut2 protein expression was greatly restored, and β-cell function and survival were highly preserved[11].

Neratinib restores glycemia in a T2D mouse model. Obese diabetic Lepr$^{db/db}$ mice (db/db, Fig. 8) become severely diabetic and exhibit β-cell apoptosis and dysfunction[40] together with islet upregulation of activated MST1[11] at the age of 10 weeks. To test whether MST1 inhibition by neratinib could affect glycemia in

Fig. 6 Neratinib improves glycemia, insulin secretion, and β-cell survival in the MLD-STZ-mouse model of type 1 diabetes. C57Bl/6J mice were injected with 40 mg/kg streptozotocin or citrate buffer for 5 consecutive days. Neratinib or vehicle was daily i.p. injected at a concentration of 5 mg/kg starting 3 h before the first STZ injection throughout the whole experiment of 35 days. **a** Random fed blood glucose measurements after the first STZ injection (day 0) over 35 days and **b** intraperitoneal glucose tolerance test (ipGTT); (respective area-under-the curve (AUC) analyses are shown in the right insets). **c**, **d** Intraperitoneal insulin tolerance test (ipITT). In **d**, basal glucose values were normalized to 100%. **e** Insulin secretion measured from retro-orbital blood draw during an ipGTT measured before (0 min) and 15 min after glucose injection; data are expressed as the ratio of secreted insulin at 15 min/0 min (stimulatory index). **f** The ratio of secreted insulin and glucose is calculated at fed state. **g**–**i** Mice were killed at day 35. **g** β-cell mass (given as percentage of the whole pancreatic section from ten sections spanning the width of the pancreas) and quantitative analyses from triple staining for **h** TUNEL or **i** Ki67, insulin, and DAPI expressed as percentage of TUNEL- or Ki67-positive β-cells ±SEM. An average number of 2331 (**h**), or 26369 (**i**) β-cells were counted. $n = 9$ mice/group for (**a**); $n = 4$–12 for (**b**); $n = 5$–15 for (**c**) and (**d**); $n = 12$–19 for (**e**); $n = 8$–14 for (**f**); $n = 4$–9 for (**g**); $n = 4$–11 for (**h**); and $n = 6$–11 mice/groupfor (**i**); all from three independent experiments). Data show means ± SEM. $^{*}p < 0.05$ STZ compared with vehicle-injected mice, $^{**}p < 0.05$ STZ compared with STZ-Nera injected mice; by one-way ANOVA with Bonferroni corrections for (**a**, **b**); by Student's *t* tests for (**e**–**i**). Source data are provided as a Source Data file

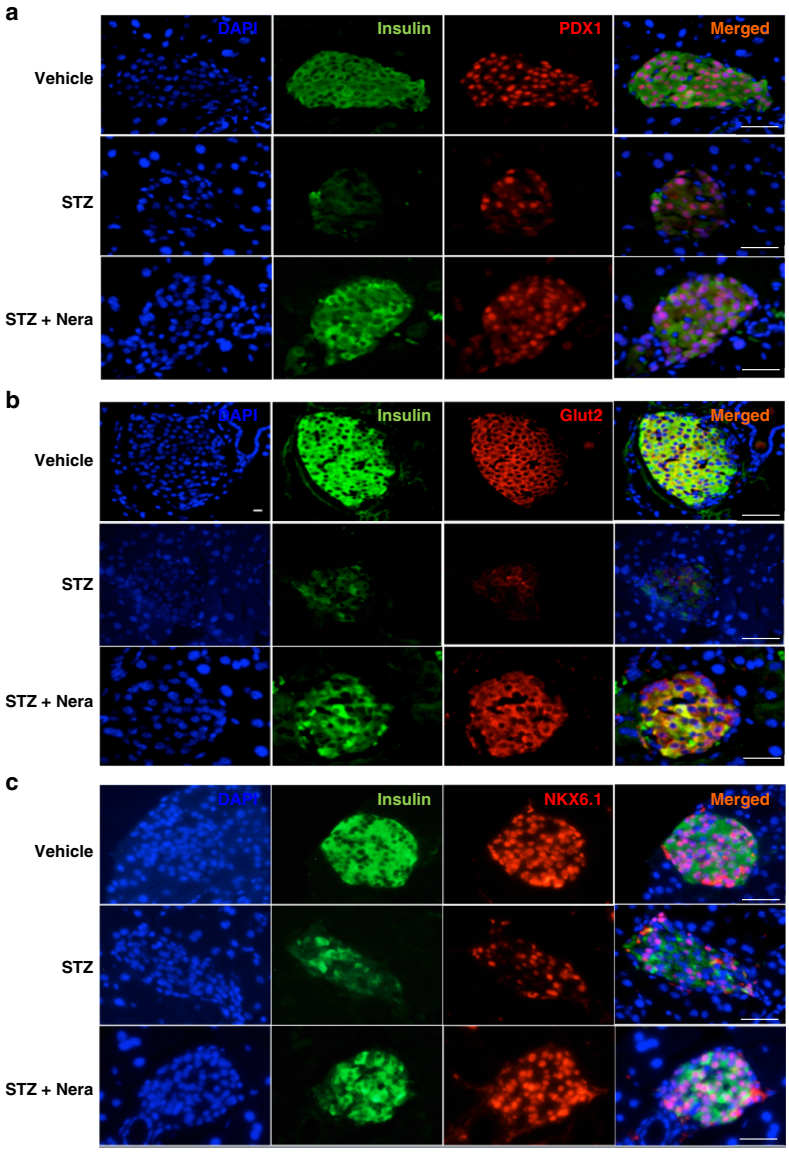


Fig. 7 Neratinib restores PDX1, NKX6.1, and Glut2 expression. Representative triple stainings for PDX1 (red, **a**), Glut2 (red, **b**), or NKX6.1 (red, **c**), insulin (green), and DAPI (blue) are shown from vehicle-, STZ-, and STZ/Nera-treated mice ($n = 8$, 4, 9 & 9 mice/group). Scale bar, 100 μm

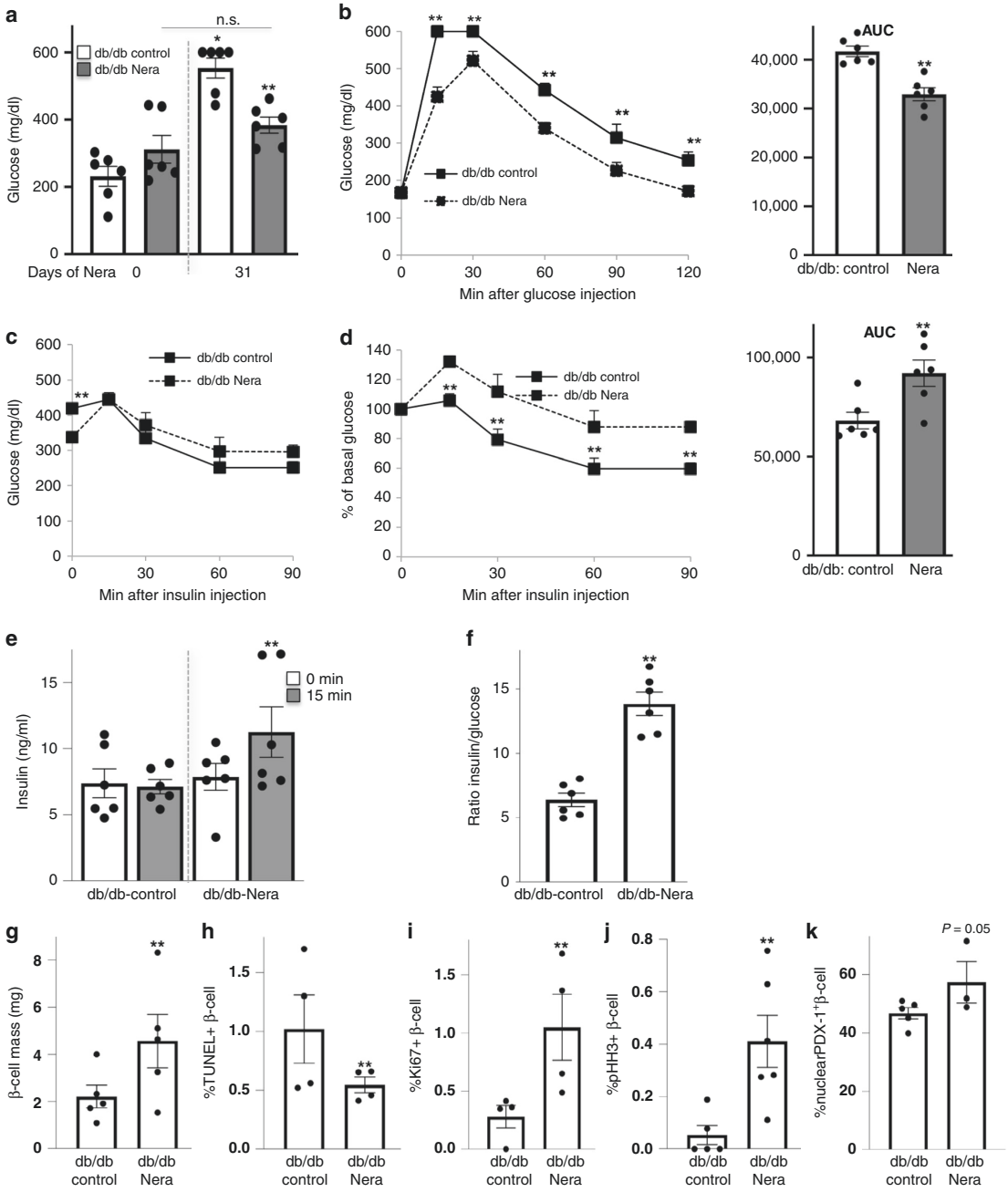


Fig. 8 Neratinib improves glycemia, insulin secretion, and β-cell survival in obese db/db mouse model of type 2 diabetes. Obese diabetic Lepr$^{db/db}$ mice on the C57BLKS/J background (db/db) were randomized in two groups at the age of 6 weeks, and then, neratinib or vehicle was daily i.p. injected at a concentration of 5 mg/kg throughout the whole experiment of 31 days. **a** Random fed blood glucose measurements before and after 31 days of Neratinib or vehicle injection (last day of the study). **b** Intraperitoneal glucose tolerance test (ipGTT). **c**, **d** Intraperitoneal insulin tolerance test (ipITT). In **d**, basal glucose values were normalized to 100% (respective area-under-the-curve (AUC) analyses for b and d are shown in the right insets). **e** Insulin secretion during an ipGTT measured before (0 min) and 15 min after glucose injection. **f** The ratio of secreted insulin and glucose is calculated at fed state. Data are representative of six mice per group ($n = 6$ for (**a**–**f**)). **g**–**j** Mice were killed at day 31. **g** β-cell mass (given as percentage of the whole pancreatic section from ten sections spanning the width of the pancreas; $n = 5$ mice/group) and quantitative analyses from triple stainings for (**h**) TUNEL, (**i**) Ki67, (**j**) pHH3, and (**k**) nuclear PDX1 expression, insulin, and DAPI expressed as percentage of TUNEL-, Ki67-, pHH3-, or nuclear PDX1-positive β-cells ± SEM. An average number of 13667 (**h**), 5937 (**i**), 7108 (**j**), or 4330 (**k**) β-cells were counted from $n = 3$–5 (**h**, **i**, **k**) or $n = 5$–6 (**j**) mice/group. Data show means ± SEM. $^*p <$ 0.05 vehicle control at the end (10.5 weeks of age) compared with the start of the study (6 weeks of age), $^{**}p < 0.05$ db/db compared with db/db-Nera injected mice; by one-way ANOVA with Bonferroni corrections for (**a**); by Student's *t* tests for (**b**–**k**). Source data are provided as a Source Data file

the db/db model, 6-week-old obese db/db mice were treated daily with neratinib or vehicle over a period of 31 days. Neratinib treatment had no influence on body weight (Supplementary Fig. 6c). While blood glucose levels remained stable and glucose levels did not significantly increase in the neratinib-treated db/db mice after 4 weeks, they predictably rose to severe hyperglycemia (>500 mg/dL) in the vehicle-treated db/db mice (Fig. 8a). Attenuation of hyperglycemia was also evident in the intraperitoneal glucose tolerance test, in which neratinib showed lower glucose levels at all time points measured (Fig. 8b), and enhanced insulin secretion and insulin-to-glucose ratios (Fig. 8e, f). Basal glucose levels were unchanged by neratinib treatment after an overnight fast (Fig. 8b), but 20% lower in the neratinib-treated mice after a short fast of 4 h (Fig. 8c). These differences in fasting glucose prompted us to conduct an intraperitoneal insulin tolerance test, which showed that neratinib-treated mice had a modestly reduced ability to lower their glucose levels in response to insulin challenge (Fig. 8c, d), despite neratinib's improvement in the overall restoration of glucose homeostasis. At the level of the β-cell, neratinib showed increased β-cell mass (Fig. 8g), which resulted from significantly reduced β-cell apoptosis (Fig. 8h and Supplementary Fig. 8a) and increased proliferation as determined by two markers Ki67 and phospho-Histone H3 (pHH3) immunolabeling (Fig. 8i, j and Supplementary Fig. 8b, c). While less than 50% of β-cells contained PDX1 in the nucleus, nuclear PDX1 was enhanced by neratinib (Fig. 8k and Supplementary Fig. 9).

Neratinib improves β-cell survival in an ex vivo approach. While neratinib improved glycemia in db/db mice in vivo, we aimed to confirm whether this effect occurs directly in islets ex vivo. Thus, we isolated mouse islets from severely diabetic db/db mice, where β-cell apoptosis was 3.2-fold increased, compared with islets from nondiabetic db/+ littermates. Adding neratinib to the islet cultures fully restored β-cell survival (Fig. 9a, b). MST1 activation and proapoptotic BIM in islets were reversed by ex vivo neratinib treatment (Fig. 9c, d). Neratinib also enhanced nuclear PDX1 expression (Supplementary Fig. 10).

We then addressed the question whether cytokine-induced β-cell apoptosis can also be normalized by neratinib and designed another therapeutic approach by treating the islets with the cytokine mixture of IL-1β/IFNγ cytokine mixture for 48 h and then added neratinib to the culture for another 24 h. While the cytokines induced a dramatic increase in β-cell apoptosis, neratinib fully restored β-cell survival (Fig. 9e, f).

Neratinib is enriched and distributed in the pancreas. To provide further evidence that neratinib mediates the antidiabetic effects through a direct impact on the β-cell, we sought to confirm meaningful pharmacokinetic exposure of neratinib in the pancreas by using marix-assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS)[41]. Specificity of the signal was tested on a liver phantom model, where neratinib was spotted on frozen mouse liver sections at concentrations ranging from 0 to 500 pmol/µl and peaks analyzed by MALDI imaging MS (Supplementary Fig. 11a). Starting at a concentration of 50 pmol/µl, neratinib could clearly be detected on liver sections at m/z 557.2, which represents the monoisotopic peak of neratinib (Supplementary Fig. 11a). Linear regression of signal intensity to neratinib concentration could be observed until 400 pmol/µl. The mass spectrum of the neratinib standard (Supplementary Fig. 11c) showed the expected isotope distribution as the simulated spectrum for neratinib (Supplementary Fig. 11b). Note the high abundance of the third peak at m/z 559.2, which is mostly caused by the stable isotope ^{37}Cl, which occurs in nature with an abundance of 24.2% (sum formula of neratinib $C_{30}H_{29}ClN_6O_3$). This characteristic isotope distribution could also be detected in single spectra of high neratinib-intense regions in the pancreas after treatment (Supplementary Fig. 11d). A specific signal was obtained in pancreas tissue sections after a 4-week daily i.p. injection in the db/db mice with no signal evident from vehicle-treated control mice (Supplementary Fig. 11e). Neratinib was also clearly detected in pancreatic tissue 4 h after intraperitoneal (i.p.) injection of WT mice (Supplementary Fig. 11f).

Discussion

In this study, we demonstrate neratinib as the inhibitor of MST1, a previously unappreciated activity alongside the dual inhibition of HER2/EGFR that drives its clinical utility in breast cancer. We show that neratinib protects β-cells from the apoptosis-inducing effects of a complex diabetic milieu in vitro in rat INS-1E β-cells and primary human and mouse islets, and lowers hyperglycemia in vivo in two widely used rodent models of diabetes. Repurposing of FDA-approved drugs has been a topic of great interest amidst the escalating costs of new drug development, particularly in the case of diseases with high-unmet medical need, such as T1D. Our studies suggest that neratinib—shown to be safe and well-tolerated in thousands of subjects in many Phase II and III clinical trials for cancer therapy[32,42]—could have a therapeutic effect in treating diabetes. Although diarrhea, vomiting, and nausea were most common neratinib-associated adverse events, they showed no increased risk of long-term toxicity or adverse consequences[32]. The appropriateness of the tolerability profile of neratinib for patients with a non-immediate-life-threatening disease such as diabetes must be considered carefully; as such, side effects may hinder its direct use in treating patients with either T1D or T2D, and thus, have to be reinvestigated in a clinical diabetes setting. Based on our mouse studies, a short therapeutical interval of 30 days could markedly restore β-cell survival and function, and thus, maybe sufficient for therapy in patients. Especially in the obese db/db model, it becomes clear that neratinib treatment prevented the severe increase in blood glucose over time. We started the experiment, when mice were already mildly hyperglycemic (mean random glucose of all mice was 271.5 mg/dl). After 30 days of therapy, the control group showed a 2.4-fold increase in blood glucose (from 231 to 554 mg/dl), while the neratinib group had no significant blood glucose increase (312–384 mg/dl).

Besides their potent action in cancer therapy, multitarget tyrosine inhibitors have been suggested for a long time for the treatment of diabetes, in some cases driven by polypharmacology outside of the tyrosine kinase target class. For example, imatinib, which targets c-Abl, DDR1/2, c-Kit, and PDGFR; sunitinib, which targets FLT1/3/4, c-Kit, PDGFR, and VEGFR2; erlotinib and PD153035, potent and specific inhibitors of EGFR, have all been reported to have potent anti-hyperglycemic effects in preclinical as well as in several clinical case reports[43,44]. In a related compound class, the tyrosine phosphatase PTP1b inhibitor ertiprotafib was explored as a novel insulin sensitizer for T2D, based on its ability to improve fasting blood glucose and glucose tolerance in the Zucker diabetic fatty rat[45], with triglyceride and free fatty acid lowering effects mediated through inhibition of IκB kinase β[46]. With regard to EGFR inhibitors, two independent studies show profound reduction of fasting glucose levels and normalization of HbA1c in two lung cancer patients with T2D treated with the EGFR inhibitor erlotinib[47,48]. Although such cases were not reported with neratinib, one wonders whether the well-known polypharmacology of erlotinib may overlap with neratinib and thus exhibit antidiabetic effects.

EGFR signaling is associated with insulin resistance and liver, muscle, and adipose inflammation. EGFR inhibition through

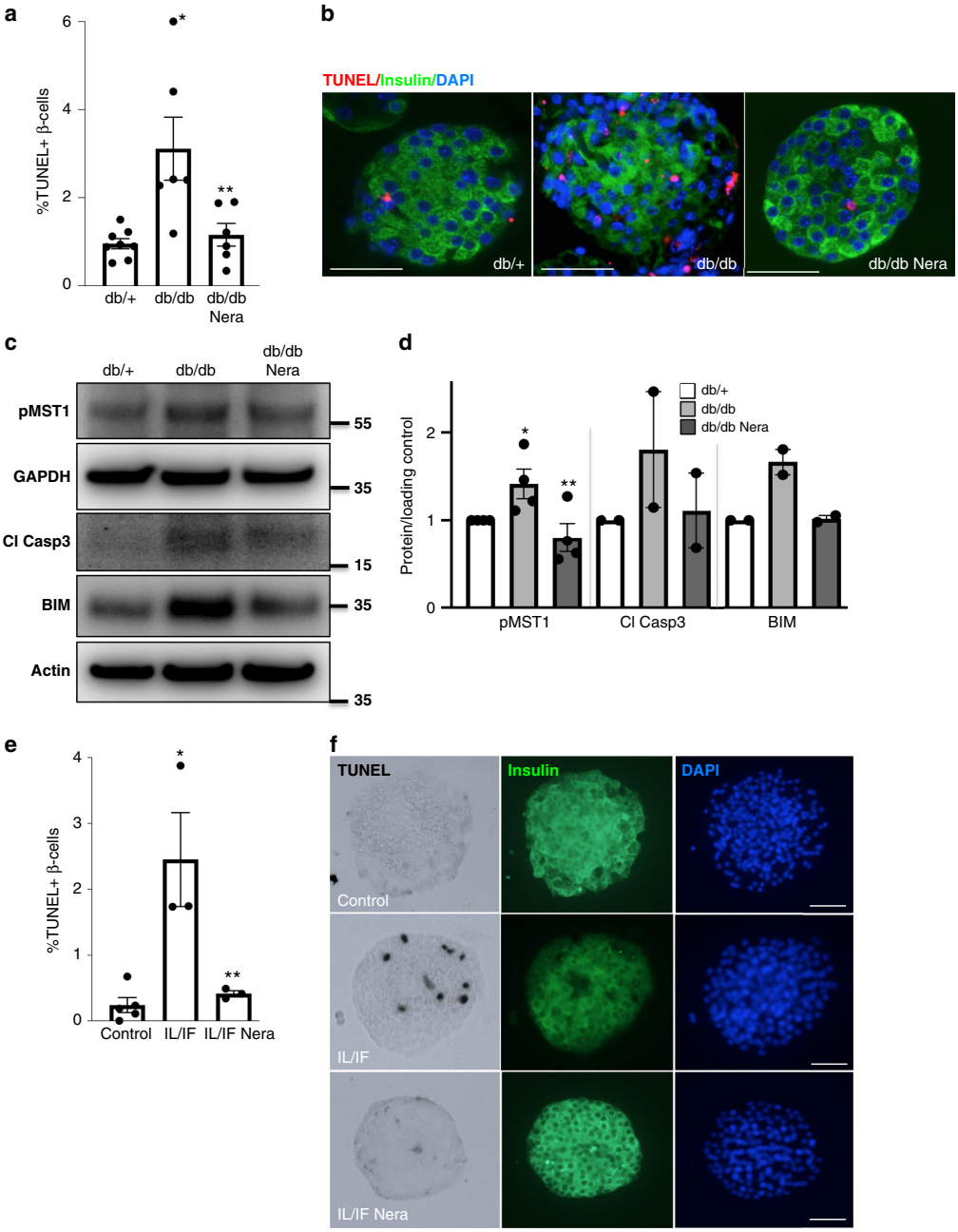


Fig. 9 Neratinib improves β-cell survival in islets in a therapeutic ex vivo approach. **a**, **b** Isolated islets from 10-week-old obese diabetic Lepr$^{db/db}$ mice or their heterozygous db/+ littermates were exposed to 10 µM neratinib for 24 h, fixed, and 2-µm sections were prepared. **a** Percentage of TUNEL-positive β-cells is shown as means ± SEM. **b** β-cell apoptosis was analyzed from islet sections by triple staining of TUNEL (red nuclei), insulin (green), and dapi (blue); scale bar, 100 µm. **c** Phospho-MST1 (pMST1; pThr183), caspase-3 cleavage, BIM and GAPDH, or actin were analyzed by western blotting shown by a representative blot (**c**) and pooled quantitative densitometry analysis (**d**). **e**, **f** Isolated islets from 2-month-old WT C57Bl/6 mice were exposed to the IL-1β/IFNγ cytokine mixture for 72 h (IL/IF); 10 µM neratinib was added to the culture for the last 24 h. **e** Percentage of TUNEL-positive β-cells is shown as means ± SEM. **f** β-cell apoptosis was analyzed from attached islet cultures by triple staining of TUNEL (black nuclei), insulin (green), and dapi (blue). Scale bar, 100 µm. All data are means ± SEM from multiple mice/condition ($n = 6$–8 for (**a**); $n = 2$–4 for (**c**); $n = 3$–5 for (**e**)). *$p < 0.05$ db/db or IL/IF to heterozygous db/+ or untreated control islets, **$p < 0.05$ neratinib to vehicle-treated islets under the same diabetogenic conditions; all by Student's *t* tests. Source data are provided as a Source Data file

tyrosine kinase inhibition was suggested as therapy of insulin resistance, because its inhibition could restore insulin sensitivity by decreasing inflammation in insulin target tissue[43]. EGFR inhibition improved tyrosine phosphorylation levels of the insulin receptor and the insulin receptor substrate in obese mice, which are classically associated with improved insulin sensitivity; however, direct analysis of insulin sensitivity was not performed in these studies[44].

Although FDA was approved for cancer therapy, further medicinal chemistry optimization to improve drug selectivity and remove its covalent linkage would be desirable to limit drug toxicity and to provide better specificity for a chronic indication like diabetes. Neratinib inhibits MST1 very potently; however, it targets many other kinases, as the development of kinase inhibitor has been challenging[38,49]. A foundation for kinase inhibitor biology and toxicity has been set by an excellent previous study, which also reported potent activity of neratinib (also formerly known as HKI-272) on MST family members[38]. Studies to improve MST1 specificity and potency are currently under way.

While neratinib is not at all selective for MST1, we believe that the underlying mechanisms of diabetes protection still derive from a direct protective effect on the β-cell mediated by MST1 and not from an effect on insulin sensitivity mediated by EGFR. Improved glucose tolerance and insulin secretion were observed in both models, while insulin sensitivity was unaffected by neratinib in the STZ model and modestly impaired in the obese insulin-resistant db/db model, which was only evident after normalization of the fasting glucose levels. Without such normalization applied, glucose levels during the insulin tolerance test were not significantly affected by neratinib, despite the significant glucose reduction in neratinib-treated mice after 4 h of fasting. Also, neratinib almost fully blocked β-cell apoptosis in human islets induced by MST1 overexpression and also had no additive effect in islets isolated from MST1-KO mice, in which apoptosis was already blocked by the genetic disruption of MST1 itself.

A general concern in targeting the reduction of β-cell apoptosis and the induction of β-cell proliferation in diabetes therapy is the potential for uncontrolled expansion of multiple cell types and oncogenic transformation. Organ-specific double knockout of MST1/2, e.g., in the liver has been shown to lead to tumor growth, because of the lack of constraints on cellular proliferation in these mice driven by the Hippo-YAP pathway[50]. Although neratinib shows some modest selectivity toward MST1 than to MST2, one of our long-term objectives will be to generate an MST1 inhibitor with greater selectivity versus MST2 than neratinib (e.g., more than 50-fold). In our previous work, we carefully explored the phenomenon of β-cell proliferation in both the global as well as β-cell-specific MST1-knockout mice. At basal normoglycemic level, there was no change in β-cell proliferation in the MST1-KO, but there was increased β-cell proliferation under conditions of STZ-induced hyperglycemia both in the global as well as β-cell-specific MST1-KO mice. Also, β-cell-specific MST1 ablation fosters β-cell compensatory hyperplasia in HFD-treated mice[11,24]. This suggests that there is no proliferative potential of MST1 inhibition under non-stressed conditions, but the capacity to overcome β-cell stress by increased proliferation and reduced β-cell apoptosis is triggered under circumstances of such stress, leading to reestablishment of glucose homeostasis. Similarly, neratinib did not affect apoptosis or proliferation in nondiabetic mice, but increased β-cell proliferation in db/db mice together with profound reduction in β-cell apoptosis, leading to β-cell mass compensation and improved glycemia. However, in the absence of CRISPR-mediated deletion of all other potential neratinib-sensitive kinases, similarly as done previously[51] we cannot formally exclude off-target effects and definitively assign MST1 as the only target of neratinib in this study.

Overall, basal β-cell proliferation is very limited in mouse islets and negligible in human islets[52]. This is probably an evolved property that protects from insulin production during long times of starvation. This proliferative incapacity can be attributed at the molecular level to the loss of YAP, the major downstream component of the Hippo-YAP pathway, which controls organ development and size[37,53–55]. YAP disappears exactly at the point of islet development, when Ngn3 becomes present in order to drive endocrine cell differentiation[37,56,57]. Therefore, MST1 inhibition in the absence of YAP expression would not lead to tumor development. The Hippo-YAP pathway acts in coordination with many other cell-size and proliferation-determining factors in development, including insulin-like growth factor (IGF1R[58]) and EGF receptor signaling (EGFR[59]). Such interaction can even be cell-autonomous, as shown in Drosophila, where YAP-EGFR crosstalk promotes proliferation in neighboring cells[60]. Thus, we cannot rule out the possibility that simultaneous EGFR inhibition could be important for limiting the oncogenic potential of MST1 and MST2 inhibition and subsequent activation of YAP directed transcription.

Several MST1 inhibitors have been identified recently[61]. Compound 9E1, the first small-molecule MST1 inhibitor identified from an organometallic library screen[27], showed strong off-target effects on other kinases such as proto-oncogene serine/threonine protein kinase PIM1 (PIM-1) and glycogen synthase kinase 3 (GSK-3β)[27], and thus did not enter any preclinical studies. MST1 inhibition by LP-945706 has anti-inflammatory efficacy in an experimental autoimmune encephalomyelitis (EAE) model[29]. The reversible and selective MST1/2 inhibitor XMU-MP-1 promotes tissue repair and regeneration by cellular proliferation induction in human liver cells and in a mouse model of liver and intestine injury[28]. It will be interesting to test the efficiency of the novel MST1 inhibitors LP-945706 or XMU-MP-1 to promote β-cell survival exposed to a complex diabetic milieu or in diabetic mice in comparison with neratinib.

This study shows the beneficial effects of the kinase inhibitor neratinib in ameliorating hyperglycemia as well as improving β-cell survival and function under diabetogenic conditions. The subject of our ongoing work in this regard is the design of neratinib-based MST1 inhibitors that exhibit enhanced potency and selectivity for MST1, with safety profiles commensurate with the chronic treatment of diabetes. The identification of neratinib as an MST1 inhibitor thus amounts to an accelerated path to a preclinical proof of concept, shown herein, as well as a firm basis for a follow-on medicinal chemistry optimization program aimed at retaining the drug-like properties of neratinib but improving upon its selectivity and safety.

Methods

Cell culture, treatment, and islet isolation. Human islets were isolated from eight pancreases of nondiabetic organ donors at PRODO Labs and at Lille University and cultured on extracellular matrix-coated dishes (Novamed, Jerusalem, Israel)[62] or on Biocoat Collagen I coated dishes (#356400, Corning, ME, USA). 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). Islets from MST1-knockout (MST1-KO) mice and their WT littermates[63] were isolated by pancreas perfusion with a Liberase TM (#05401119001, Roche, Mannheim, Germany) solution[62] according to the manufacturer's instructions and digested at 37 °C, followed by washing and handpicking. The clonal rat β-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 mM glucose, mouse islets and INS-1E cells at complete RPMI-1640 medium at 11.1 mM glucose[11]. Mouse macrophage Raw264.7 cell line was purchased from ATCC and cultured in DMEM (Gibco) supplemented with 10% FBS. Islets and INS-1E were exposed to complex diabetogenic conditions: 22.2–33.3 mM glucose, 0.5 mM palmitic acid, the mixture of 2 ng/ml recombinant human IL-1β (R&D Systems, Minneapolis, MN) + 1000 U/ml recombinant human IFN-γ (PeProTech) for 24–72 h, 100 μM H_2O_2 for 6 h, or 0.1, 1 mM thapsigargin for 6 or 24 h. Neratinib or vehicle (0.1% DMSO) was added to the cell culture 1–2 h before treatment. Palmitic acid (Sigma) was

dissolved at 10 mmol/l in RPMI-1640 medium containing 11% fatty acid–free bovine serum albumin (BSA) (Sigma) under an N2-atmosphere and added to the culture at 0.5 mM[64]. In some experiments, human or mouse islets or INS-1E cells were additionally cultured with various concentrations of neratinib (Calibr). Isolated human islets or INS-1E cells were infected with adenovirus carrying LacZ as control or MST1 or LATS2 (all Vector Biolabs), at a multiplicity of infection of 100 (human islets) or 20 (INS-1E) for 4 h[11]. Adenovirus was subsequently washed off with PBS and replaced by fresh medium with 10% FBS and the respective analysis performed after 48 h post infection.

All human islet experiments were performed in the islet biology laboratory, University of Bremen. Ethical approval for the use of human islets had been granted by the Ethics Committee of the University of Bremen. The study complied with all relevant ethical regulations for work with human cells for research purposes. Organ donors are not identifiable and anonymous; such approved experiments by using human islet cells for research are covered by the NIH Exemption 4 (Regulation PHS 398). Human islets were distributed by the two JDRF and NIH-supported approved coordination programs in Europe (Islet for Basic Research program; European Consortium for Islet Transplantation ECIT) and in the United States (Integrated Islet Distribution Program IIDP)[65].

High-throughput screening and hits confirmation. High-throughput screening targeting MST1 was conducted in low-binding 1536 microplate (Corning, NY) by using LanthaScreen Eu Kinase Binding assay. In total, 641 annotated compounds in a Kinase inhibitor library (Calibr) were screened at 1, 0.2, 0.04, 0.008, and 0.0016 µM, with staurosporine as the positive control. Compounds in 1000× DMSO stock solution were dispensed by using Echo555 liquid dispensing system (Labcyte, CA) to 1536-well microplate (Corning, NY). Kinase buffer A (ThermoFisher, MA) was prepared and added to each well. MST1 kinase (ThermoFisher) and Eu-anti-GST Antibody (ThermoFisher) was prepared at 15 nM and 6 nM, respectively. Kinase tracer 222 (ThermoFisher) was prepared at 300 nM. Each reagent was added to the microplate in equal volume. Plates were incubated at room temperature for 1.5 h in the dark, scanned on Envision plate reader with excitation at 340 nM, emission at 665 and 615 nM. Data were analyzed based on the emission ratio of 665 nm/615 nm, normalized to DMSO as negative control. The criteria of picking primary hits was ≥75% inhibition at 1 µM and ≥50% inhibition at 0.2 µM, compared with staurosporine (1 µM), which is considered 100% inhibition at 1 µM. For hit confirmation, primary hits were assayed in triplicates with 12 points in a dose-dependent manner, starting at a neratinib dose of 5 µM followed by 1:3 serial dilution.

Nanosyn kinase profiling. Neratinib was tested at Nanosyn (Santa Clara, CA) in a panel of 50 biochemical kinase assays identified in Fig. 1c at 10 µM, and later on, in a panel of 250 biochemical kinase assays (Supplementary Fig. 1a) at 3 µM in duplicate wells. A selected set of kinases where more than 90% of inhibition was observed at 3 µM was retested in dose response for neratinib and IC_{50} was determined (Supplementary Fig. 1b). The testing was performed by using microfluidics mobility shift assay technology using ATP concentrations at Km level of each kinase. Data presented as average from the two duplicate wells.

Cytotoxicity CellTiter-Glo® assay. INS-1E cells were treated with compounds in a dose-dependent manner in 384-well microplates (Corning, NY) at 10^4 cells/well in 25 µL of complete grow medium. After 24 h of compound treatment, 5 µL of Celltiter-Glo® reagent (Promega, WI) was added to each well. Assay plates were shaken vigorously for 1 min at RT to achieve completed cell lysis. Luminescence intensity was detected on Envision plate reader (Perkin Elmer, MA).

Caspase-3 activation Nucview assay. INS-1E cells were treated with compounds in a dose-dependent manner in 384-well microplates (Corning, NY) at 10^4 cells/well. Apoptosis was induced after 24 h of compound treatment by 0.1 µM Thapsigargin (Torics, Bristol, United Kingdom) with caspase-3 substrate, Nucview 488 (Biotium, CA) in the treatment. Sixteen hours later, cells were fixed in 3% paraformaldehyde (Electron Microscopy Sciences, PA) and stained with Hoechst33342 (ThermoFisher). Data analysis was based on the fluorescence intensity of Nucview488 and Hoechst33342. Similarly, in caspase-3 activation assay induced by the cytokine mixtures in high-glucose conditions, INS-1E cells were exposed up to 6.7 µM of neratinib for 2 h followed by 16 h of induction in 100 ng/mL of TNFα and 200 ng/mL of IFNγ with 33 mM glucose in assay medium. Caspase-3 activity was evaluated through Nucview488 and Hoechst33342 staining.

RT-PCR assay. Mouse macrophage Raw264.7 cells were treated with vehicle (0.1% DMSO) or neratinib in triplicates at different concentrations for 2 h, followed by 100 ng/mL LPS stimulation for 4 h. Cells were washed and RNA isolated by using RNeasy Mini Kit (Qiagen). One microgram of cDNA of each treatment sample was synthesized by using SuperScript III First-Strand Synthesis (Invitrogen). Taqman mouse primers were purchased from ThermoFisher: TNFα (Catalog No. Mm00443258_m1), IL-6 (Catalog No. Mm00446190_m1), IL-1β (Catalog No. Mm00434228_m1), and GAPDH as endogenous housekeeping control (Catalog No. Mm99999915_g1). The qPCR reaction was set up for TNFα, IL-6, IL-1β, and GAPDH individually, with technical triplicates for each gene per treatment sample and performed by the Applied Biosystems ViiA 7 real-time PCR system. The ΔΔCT method was used to analyze the relative changes in gene expression.

CETSA assay. For the CETSA assay[39], INS-1E cells were treated with 5 µM neratinib or canertinib for 2 h in the CO_2 incubator at 37 °C in a 6-well plate. Thereafter, cells were pelleted at 200 g for 4 min and resuspended in PBS supplemented with phosphatase and protease inhibitor cocktail at the cell density of 3 Mill./100 µl. Each cell suspension was distributed into five 0.2-ml PCR tubes with 100 µl of cell suspension per tube. PCR tubes were heated at their designated temperature (43–55 °C) on a thermal cycler for 3 min, incubated at room temperature for 3 min, and snap-frozen in liquid nitrogen. Cell lysates were prepared by freezing–thawing the samples in liquid nitrogen twice, and soluble MST1 was detected by western blot analysis.

Animals. For the MLD–STZ experiment, 8–10-week-old male C57BL/6J mice were i.p. injected with streptozotocin (STZ; 40 mg/kg; Sigma) freshly dissolved in 50 mM sodium citrate buffer (pH 4.5) or citrate buffer as control for 5 consecutive days (referred to as multiple low doses/MLD–STZ). Obese diabetic $Lepr^{db/db}$ mice on the C57BLKS/J background (db/db) were obtained from Charles River at the age of 5 weeks and randomized in 2 groups at the age of 6 weeks. Neratinib or vehicle (30% PEG400/0.5% Tween80/5% propylene glycol in NaCl) was daily i.p. injected at a concentration of 5 mg/kg starting 3 h before the first STZ injection or at 6 weeks of age (db/db) throughout the whole experiment. Random blood was obtained from the tail vein of non-fasted mice, and glucose was measured by using a Glucometer (Freestyle; TheraSense Inc., Alameda, CA). Mice were killed at the end of the experiment, and their pancreases were isolated. Throughout the whole study, body weight was measured weekly. 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 NIH animal care guidelines, §8 German animal protection law, German animal welfare legislation, and with the guidelines of the Society of Laboratory Animals and the Federation of Laboratory Animal Science Associations. All protocols were approved by the Bremen Senate (Senator for Science, Health, and consumer protection), and we have complied with all relevant ethical regulations for animal testing and research.

Glucose and insulin tolerance tests, insulin secretion. For intraperitoneal glucose tolerance tests, mice were fasted 12 h overnight and injected i.p. with glucose (40%; B.Braun, Melsungen, Germany) at a dose of 1 g/kg body weight. Blood samples were obtained at time points 0, 15, 30, 60, 90, and 120 min for glucose measurements by using a Glucometer. For i.p. insulin tolerance tests, mice were injected with 0.75 U/kg body weight recombinant human insulin (Novolin, Novo Nordisk) after 4–5-h fasting, 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/kg) and measured by using ultrasensitive mouse Elisa kit (ALPCO Diagnostics, Salem, NH).

Immunohistochemistry. Mouse pancreases were dissected and fixed in 4% formaldehyde at 4 °C for 12 h before embedding in paraffin[62]. Two-four-micrometer sections were deparaffinized, rehydrated, and incubated overnight at 4 °C with anti-PDX-1 (Abcam; #47267), anti-Glut2 (Chemicon; #07-1402), anti-Ki67 (Dako; #M7249), anti-phospho-Histone H3 (Ser10; Merck #06-570), and anti-NKX6.1 (DSHB, University of Iowa #F55A12[66]) in combination with TSA (Invitrogen #T30955), or for 2 h at room temperature with anti-insulin (Dako; A0546) antibodies (all at a dilution of 1:100, except anti-PDX-1, which was diluted 1:400) followed by fluorescein isothiocyanate (FITC)- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted with Vectashield with 4′6-diamidino-2-phenylindole (DAPI) (Vector Labs). β-cell apoptosis was analyzed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (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 by using a Nikon MEA53200 (Nikon GmbH, Dusseldorf, Germany) microscope, and images were acquired by 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 by using a Nikon MEA53200 (Nikon GmbH, Dusseldorf, Germany) microscope, and images were acquired by using NIS-Elements software (Nikon). β-cell mass was obtained by multiplying the β-cell fraction by the weight of the pancreas.

Western Blot analysis. At the end of the incubation periods, islets and INS-1E cells were washed in ice-cold PBS and lysed in RIPA lysis buffer containing 50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS 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 blocking by 2.5% milk (Cell Signaling) and 2.5% BSA, membranes were incubated overnight at 4 °C with rabbit anti-cleaved caspase-3 (#9664), rabbit anti-PARP (#9532), rabbit anti-cleaved PARP (rat specific #9545), rabbit anti-phospho YAP(S127) (#4911), rabbit anti-LATS2 (#5888), rabbit anti-tubulin (#2146), rabbit anti-GAPDH (#2118), rabbit anti-β-actin (#4967) (all Cell Signaling Technology), and rabbit anti-PDX1 (#47267) and rabbit anti-p-MST1 (#79199) (both from Abcam) antibodies, all at a dilution of 1:1000, followed by horseradish-peroxidase-linked anti-rabbit IgG (Jackson). Membrane was developed by using a chemiluminescence assay system (Pierce) and analyzed using DocIT®LS image acquisition 6.6a (UVP BioImaging Systems, Upland, CA, USA). Uncropped and unprocessed scans of all Western blots are available in the Source Data file.

In vitro luciferase assay. INS1-E cells were transfected with LATS-BS firefly luciferase reporter constructs by using jetPRIME transfection reagent (PolyPlus, Illkirch, France). pCDNA3.1neo-NLucYAP15 and pCDNA3.1neo-14-3-3-CLuc was a gift from Xiaolong Yang (Addgene plasmid # 107610; http://n2t.net/addgene:107610; RRID:Addgene_107610)[35]. As internal transfection control, pRL-Renilla luciferase control reporter vector (Promega) was co-transfected into each sample. Twenty-four hours after transfection, cells were transduced with Ad-h-LacZ, or Ad-h-LATS2 and Ad-h-MST1 (Vector Biolabs) for another 48 h. Neratinib or canertinib (10 μM) was added for the last 24 h. Thereafter, Western blot analysis (see above) and luciferase assay was performed by using Dual-Luciferase Reporter Assay System (Promega)[11] in a parallel set of experiments. Luciferase signal was calculated based on the ratio of luciferase activity of LATS-BS to control reporter vector.

Marix-assisted laser desorption ionization. MALDI imaging mass spectrometry (MALDI imaging MS) was performed on pancreas, liver, colon, stomach, kidney, heart, and brain tissue sections from WT C57BL/6J and db/db mice in triplicates. Neratinib distribution in the pancreas was studied after neratinib treatment for five days with a dosage of 5 mg/kg neratinib in WT control mice or after the 31-day treatment period in db/db mice; animals were killed 4 h after the last treatment. For MALDI imaging MS, 10-μm cryo sections were cut with a cryo-microtome (CM1860, Leica Biosystems, Nussloch, Germany) and mounted on indium–tin-coated conductive glass slides (Bruker Daltonics, Bremen, Germany). The matrix (HCCA in 50% ACN, 0.5% TFA) was applied with the ImagePrep Device (Bruker Daltonics), and MALDI spectra were recorded by using a Bruker autofleX speed mass spectrometer in positive reflector mode with a mass range of 400–1400 m/z. A large-size laser diameter was used with a lateral resolution of 100 μm, and 500 laser shots per pixel were accumulated with the random walk option set to 100 shots per position. For data analyses, the unprocessed raw data were imported into the Software SCiLS Lab, version 2016b (SCiLS GmbH, Bremen, Germany). The dynamic range of the neratinib signal was analyzed by using drug standards (0–500 pmol/μl). Standards were spotted on mice liver cryo-sections and the spectral intensity was plotted.

Statistical analysis. Samples in different experiments were evaluated in a randomized manner by six investigators (D.A., A.D., K.A., R.H., B.L. and SG) who were blinded to the treatment conditions (Fig. 3c, d; 4d–g; 6g–i; 7g–j; 8a, b, e, f). Data are presented as means ± SEM unless otherwise stated with the number of independent individual experiments (biological replicates) or analyzed mice presented in the figure legends. Mean differences were tested by Student's *t* tests. ANOVA for multiple group comparisons with Bonferroni corrections was performed for data in Figs. 6a, b and 7a. *P* values < 0.05 were considered statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. Source data are provided as a Source Data file. All additional data are available from the corresponding authors upon reasonable request.

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Author contributions

K.M., A.A. and M.T. conceived the project. K.M. and A.A. supervised and designed the experiments and analyzed the data in Figs. 2–4, 5b, c, 6–9, and Supplementary Figs. 4, 6b, c, and 7–10. M.T., W.S., P.G.S., and A.K.C. supervised and designed the experiments in Figs. 1, 5a, and Supplementary Figs. 1–3, 5, and 6a. S.L., S.Y., T.B. and M.S. performed the screening and medicinal chemistry experiments and analyses. K.A., A.D., B.L., R.H., D.A., S.L., S.Y. and S.G. performed the mouse experiments and analyses as well as immunostaining and analysis. KA performed the bioluminescence assay and analysis. S.Z. performed the CETSA assay and analysis. J.O., L.H.L. performed the MALDI analysis. B.L., S.A. and K.A. performed Western blots for the INS-1E as well as mouse and human islet experiments. V.N.T. and S.J. performed PK analyses. K.M., A.A., N.R., W.S. and M.T. wrote the paper. All authors critically reviewed the paper for important intellectual content and approved the final version to be published.

Competing interests

The authors declare no competing interests.

Additional information

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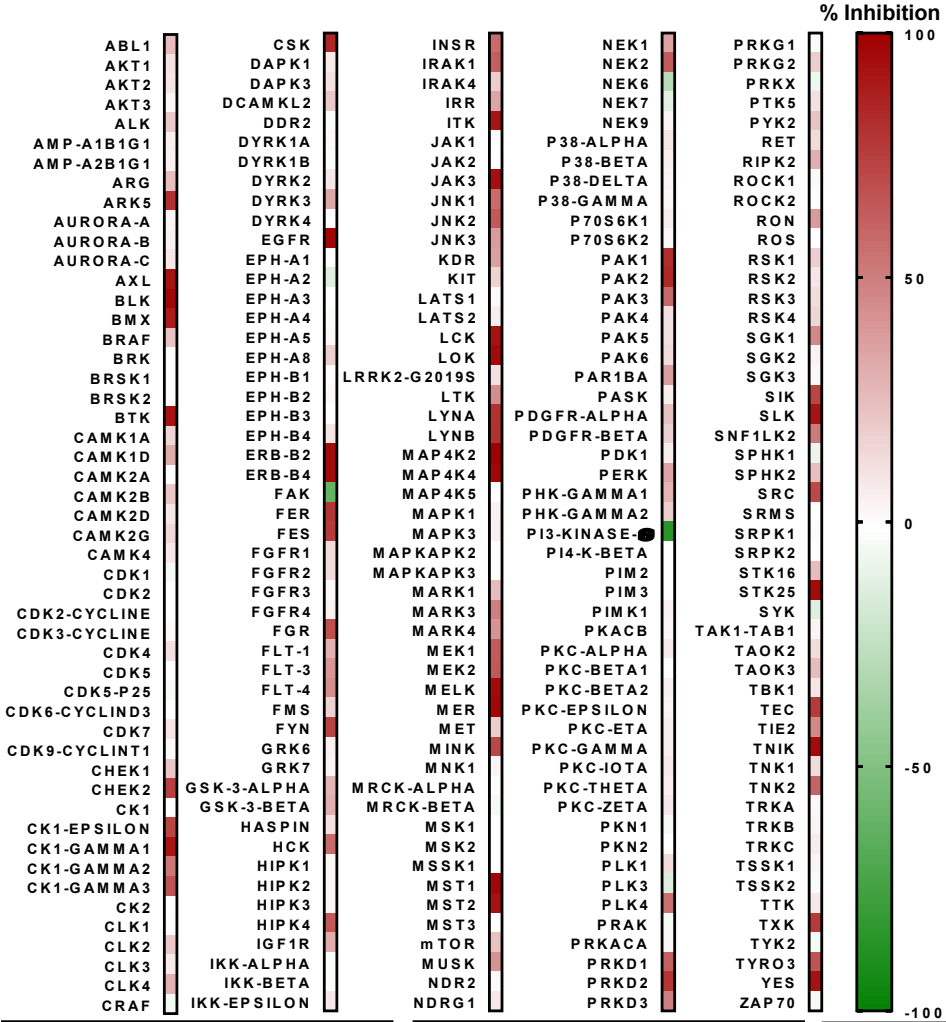
Supplementary Figures

Neratinib Protects Pancreatic Beta Cells in Diabetes

Ardestani et al.

Supplementary Figure 1

a

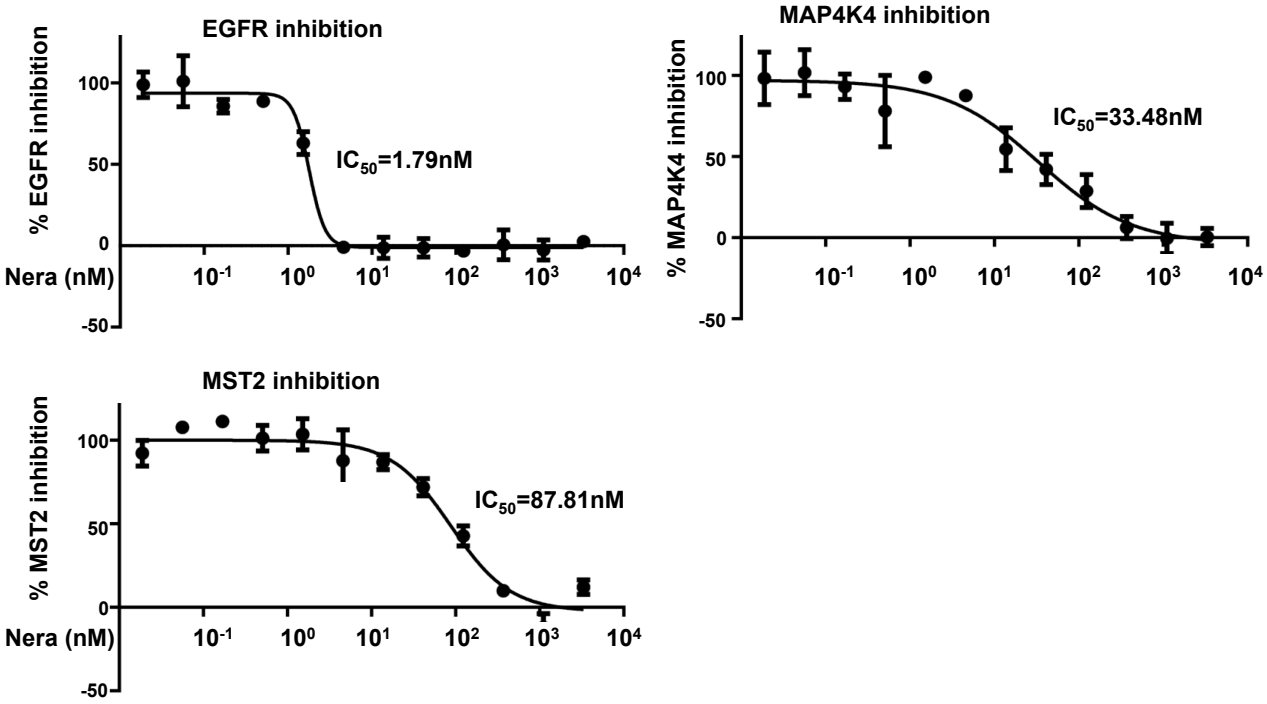


b

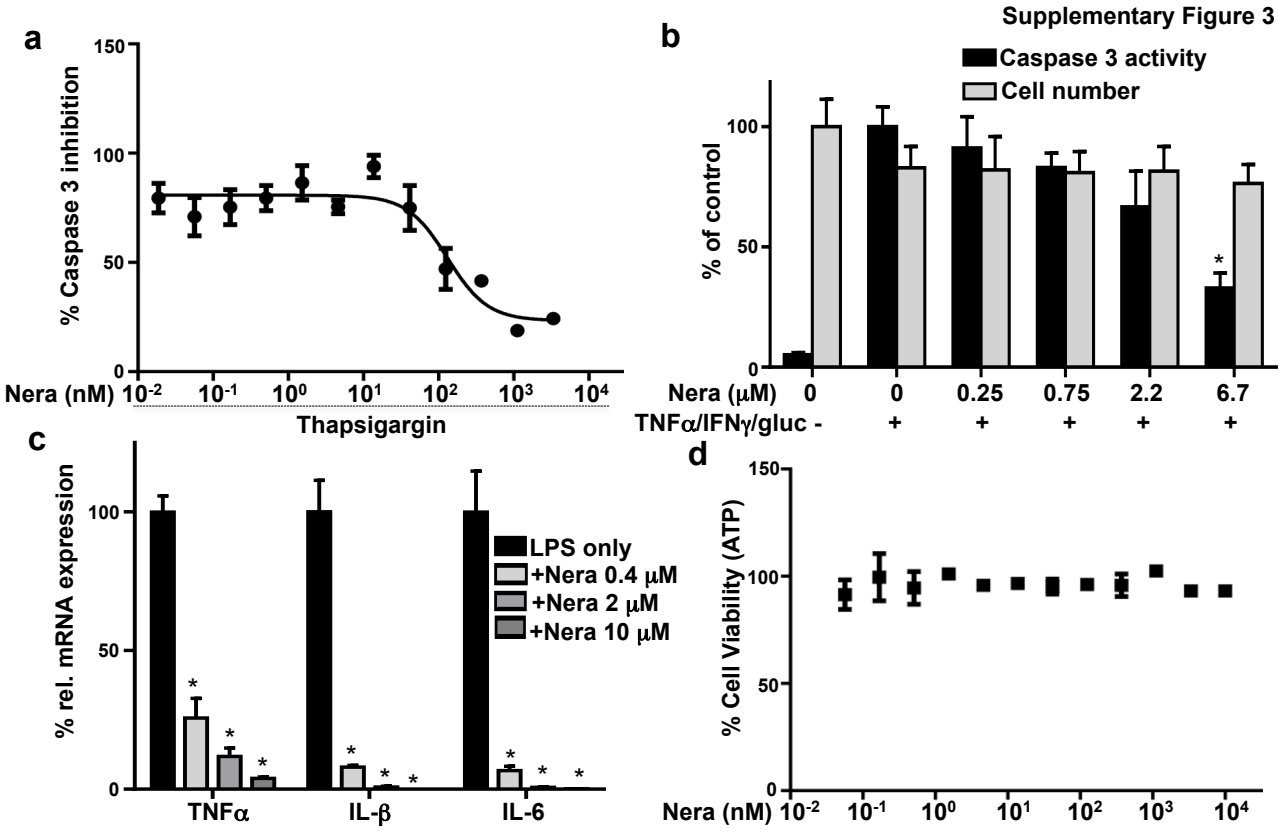
Kinases	Neratinib (IC_{50} uM)	Kinases	Neratinib (IC_{50} uM)	Kinases	Neratinib (IC_{50} uM)
IRAK1	2.48	PAK2	0.408	TNIK	0.081
LCK	0.066	PAK3	1.5	YES	0.087
LOK	0.066	PAK4	>20	ARK5	0.583
LYNA	0.379	PAK5	>20	AXL	0.185
LYNB	0.304	PAK6	>20	CHEK2	0.937
MAP4K2	0.102	PHK1	5.85	EGFR	0.0003
MAP4K4	0.062	MST1	0.091	FES	1.0
MAP4K5	0.0001	MST2	0.21	HCK	2.75
MINK	0.943	MST3	0.005	HIPK1	>20
MEK1	1.49	SIK	1.05	HIPK4	1.66
MEK2	1.2	SLK	0.177	MARK4	7.36
MER	0.026	SRC	0.727	BLK	0.016
PAK1	0.609	STK25	0.05		

Supplementary Figure 1. Results of kinase profiling for Neratinib by Nanosyn. (A) Neratinib was assayed at 3 μM in duplicate wells against 250 biochemical kinase assays. % inhibition was determined for each assay. (B) Neratinib was assayed in a 12 points dose-dependent serial concentration and IC_{50} was determined against 38 biochemical kinases. Related to Figure 1.

Supplementary Figure 2

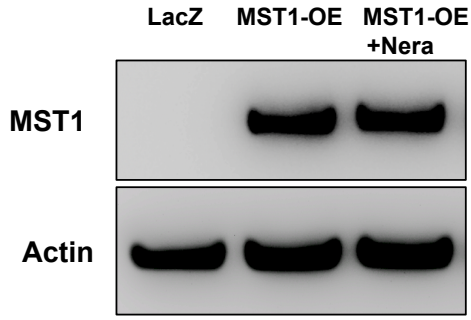


Supplementary Figure 2. Neratinib's effect on EGFR, MAP4K4 and MST2 inhibition. Biochemical dose response of EGFR, MAP4K4 and MST2 inhibition by neratinib. Data show means ±SEM from 3 independent experiments (n=3). Related to Figure 1.



Supplementary Figure 3. Neratinib protects INS-1E β-cells from ER and cytokine induced stresses. (A) Caspase-3 activation induced by thapsigargin mediated ER stress. INS-1E cells were treated with compounds in a dose-dependent manner at 10^4 cells/well. Apoptosis was induced after 24 hours by 0.1 μM thapsigargin with caspase-3 substrate Nucview488. 16 hours later, cells were fixed in 3% paraformaldehyde and stained with Hoechst33342. Data analysis is based on the fluorescence intensity of Nucview 488 and Hoechst33342, with normalization to cell cytotoxicity, which was evaluated through Celltiter-Glo. (**B**) Caspase-3 activation induced by cytokine mixture at high glucose. INS-1E cells were exposed up to 6.7μM of neratinib for 2h followed by 16h of induction in 100 ng/mL of TNFα and 200 ng/mL of IFNγ with 33 mM glucose in assay medium. Caspase-3 activity was evaluated through Nucview488 and Hoechst33342. **(C)** Anti-inflammatory effect of neratinib through gene expression assay (RT-PCR). Mouse macrophage Raw264.7 cells were treated with neratinib in different concentrations for 2 hours, and followed by 100 ng/mL LPS stimulation for 4 hours. Cells were harvested and gene expression of TNFα, IL-6 and IL-1β were analyzed through qRT-PCR. **(D)** Cytotoxicity of neratinib evaluated by CellTiter-Glo®. INS1E cells were treated with neratinib in a dose-dependent manner at 10^4 cells/well. 24 hours later, Celltiter-Glo® reagent was added to each well and luminesence intensity was detected on plate reader. Data show means±SD from 3 independent experiments (n=3). *p<0.01 neratinib compared to vehicle treated cells. Related to Figure 2.

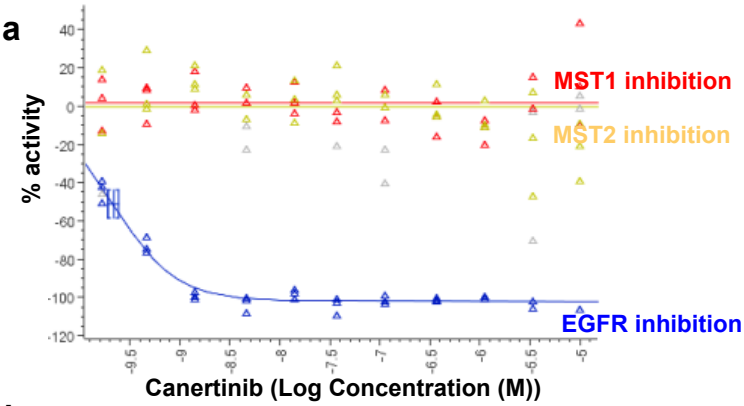
Supplementary Figure 4



Supplementary Figure 4. MST1 overexpression in human islets. Human islets were infected with Ad-LacZ (control) or Ad-MST1 adenoviruses and exposed to 10 μM neratinib for 48h; successful MST1 overexpression is shown by western blot analysis. Related to Figure 3.

Supplementary Figure 5

a

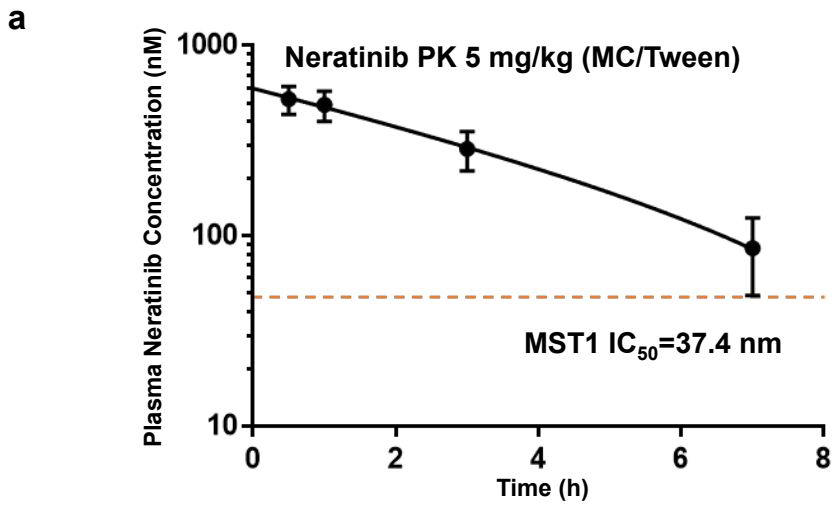


b

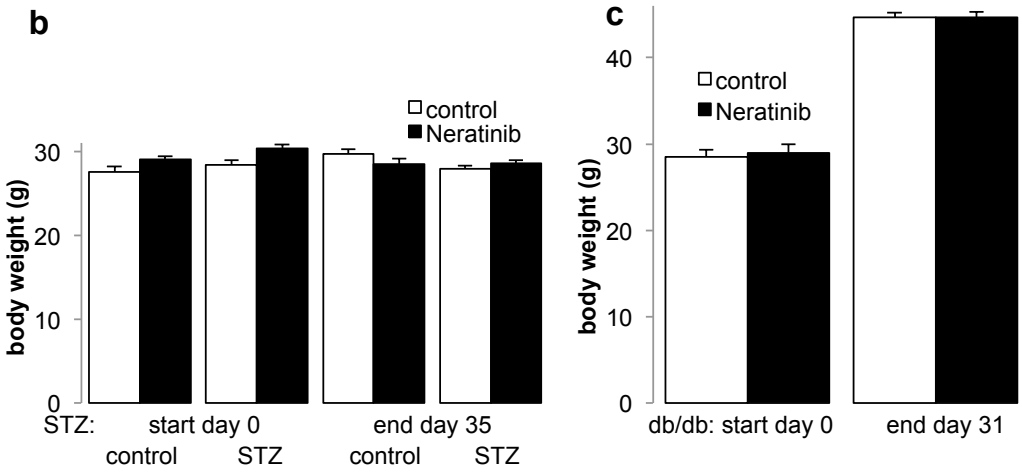
	EGFR (IC_{50})	MST1 (IC_{50})	MST2 (IC_{50})
Canertinib (nM)	0.21	> 10,000	> 10,000

Supplementary Figure 5. Canertinib's effect on EGFR, MST1 and MST2 inhibition. Biochemical kinase assays in dose response to EGFR (blue), MST1 (red) and MST2 (yellow) inhibition by canertinib **(A)** and IC_{50} summary table **(B).** Data show means from 3 independent experiments (n=3). Related to Figure 5.

Supplementary Figure 6

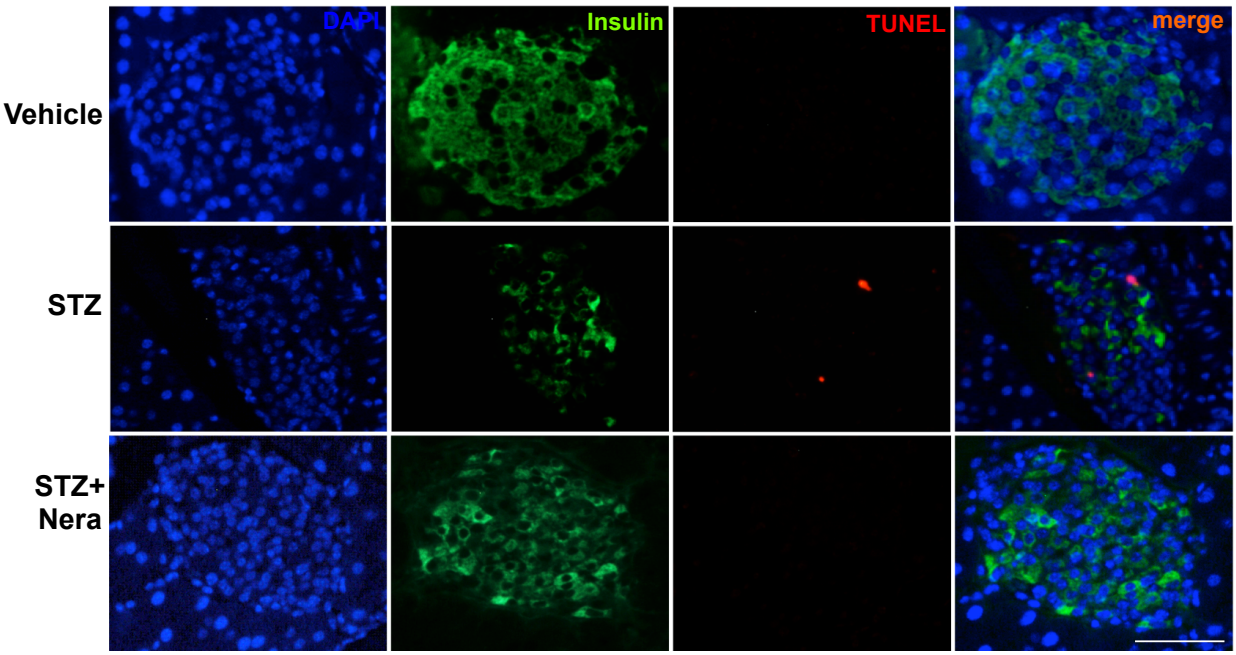


Animal	$t_{1/2}$	C_{max}	T_{max}	AUC_{0-24}	AUC_{LAST}	AUC_{INF}	Cl	MRT	V_D
	(hr)	nM	(hr)	(hr*nM)	(hr*nM)	(hr*nM)	(mL/min/kg)	(hr)	(L/kg)
1	4.0	468.6	0.5	1621.8	1621.8	2364.4	253.1	2.7	86.9
2	1.9	556.6	1.0	1967.1	1967.1	2153.7	277.9	2.3	46.7
3	1.9	623.0	0.5	2125.2	2125.2	2298.5	260.4	2.3	43.1
AVERAGE	2.6	549.4	0.7	1904.7	1904.7	2272.2	263.8	2.4	58.9
ST DEV	1.2	77.4	0.3	257.4	257.4	107.8	12.7	0.2	24.3
CV%	0.5	0.1	0.4	0.1	0.1	0.0	0.0	0.1	0.4



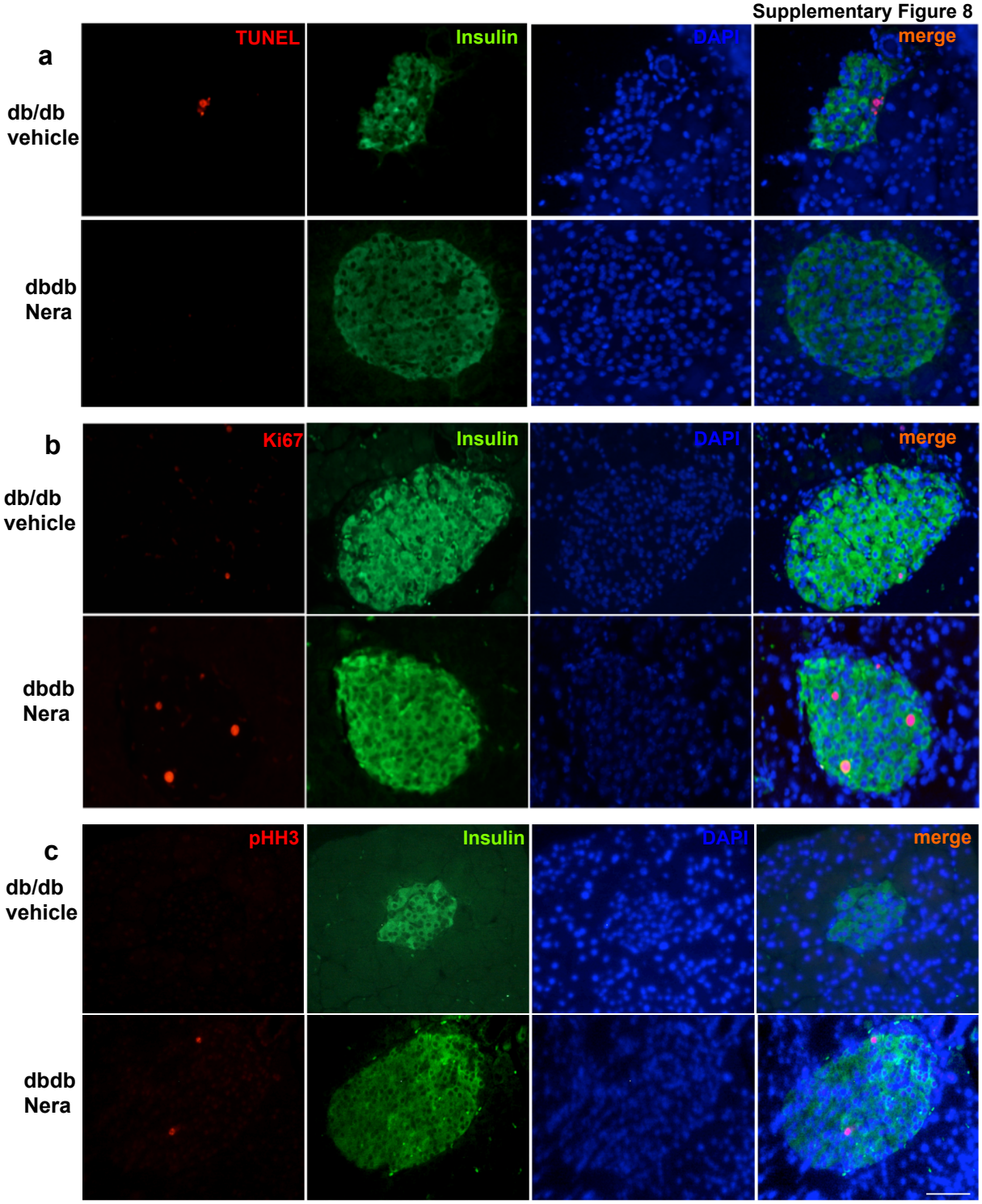
Supplementary Figure 6. (a) Neratinib's IP PK profile in mice and body weights. Neratinib was dosed at 5 mg/kg IP to mice (n=3) which were fasted overnight. The compound was administered in 30% PEG400: 0.5% Tween80: 5% propylene glycol in saline through a single dose. Plasma samples were collected at 30 min., 1, 3 and 7 h post dosing and analyzed by LC-MS to determine the plasma Neratinib concentration. **(b,c)** Mean body weights of all mice in the study from the MLD-STZ mice and db/db mice. Related to Figures 6 and 7.

Supplementary Figure 7



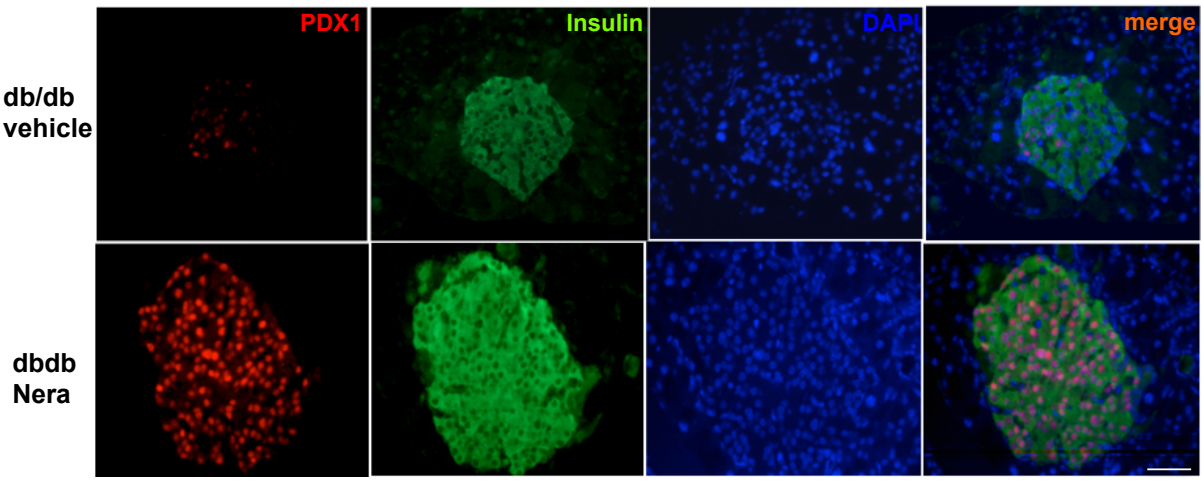
Supplementary Figure 7. Neratinib restores β-cell survival in MLD-STZ-induced diabetes. Representative triple-stainings of pancreatic sections for TUNEL (red), insulin (green) and DAPI (blue) shown from MLD-STZ diabetic mice (STZ) treated with neratinib or vehicle throughout the whole experiment of 35 days and their control (vehicle). Quantification of data are shown in Figure 6h. Related to Figure 6. Scale bar, 100 μm.

Supplementary Figure 8



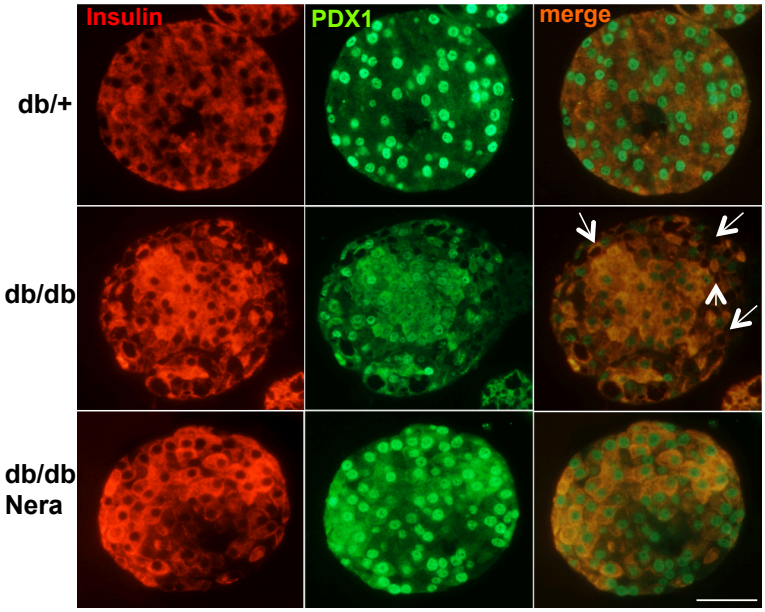
Supplementary Figure 8. Neratinib restores β-cell survival in db/db mice. Representative triple-stainings of pancreatic sections for TUNEL (a), Ki67 (b) and pHH3 (c) (red), insulin (green) and DAPI (blue) shown from db/db diabetic mice (dbdb) treated with neratinib or vehicle throughout the whole experiment of 31 days. Quantification of data are shown in Figure 8h-j. Related to Figure 8. Scale bar, 100 µm.

Supplementary Figure 9



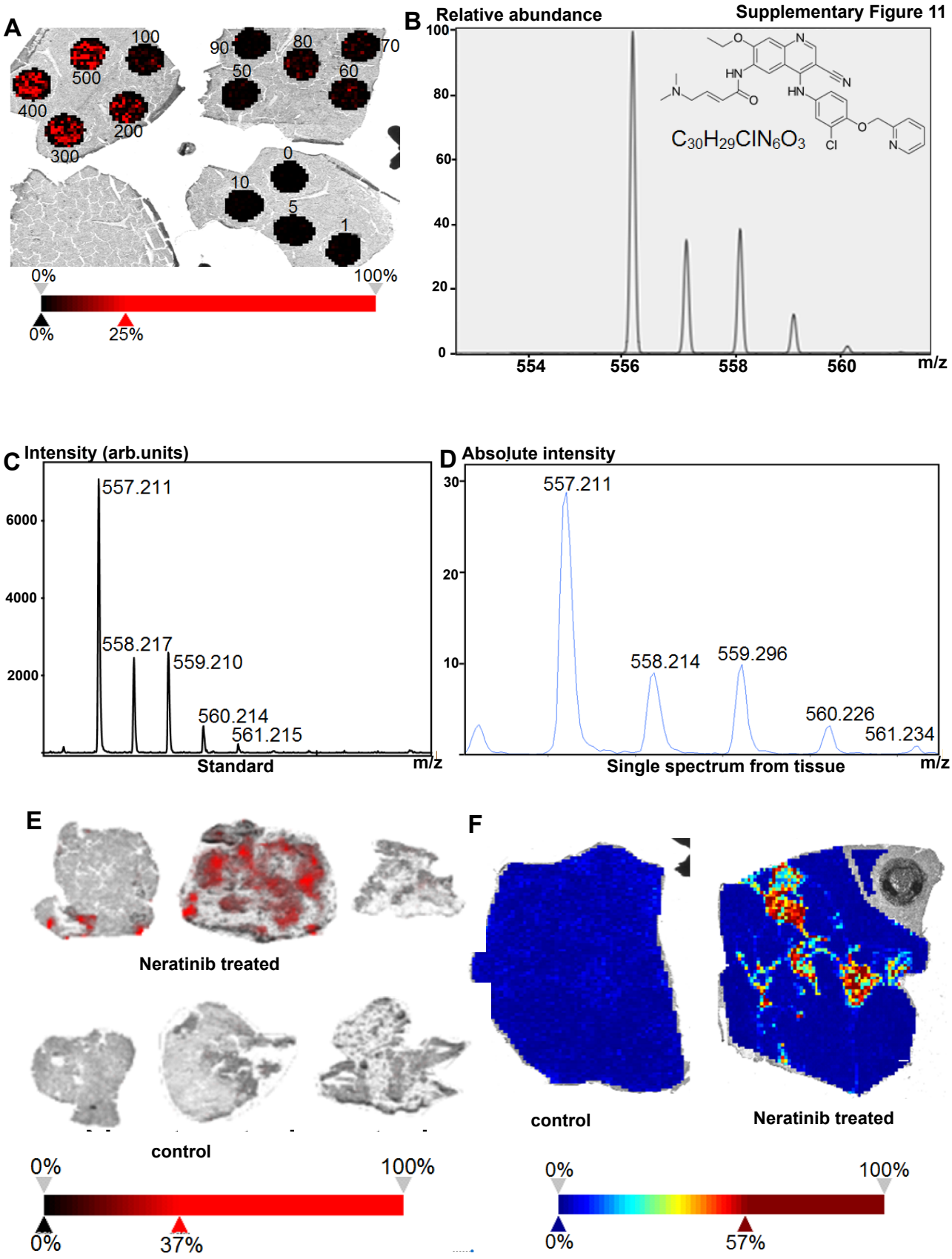
Supplementary Figure 9. Neratinib restores PDX1 in db/db mice. Representative triple-stainings of pancreatic sections for PDX1 (red), insulin (green) and DAPI (blue) shown from db/db diabetic mice (dbdb) treated with neratinib or vehicle throughout the whole experiment of 31 days. Quantification of data are shown in Figure 8k. Related to Figure 8. Scale bar, 100 µm.

Supplementary Figure 10



Supplementary Figure 10. Neratinib improves PDX1 nuclear localization in db/db mouse islets ex vivo. Representative double-stainings for PDX1 (green) and insulin (red) shown from isolated islets from 10-week old heterozygous db/+ mice or db/db littermates exposed to vehicle or to 10 μM neratinib for 24h. Representative microscopical images are shown. White arrow point to PDX1 deficient nuclei in db/db mouse islets. Related to Figure 9. Scale bar, 100 μm

Supplementary Figure 11



Supplementary Figure 11. Neratinib is enriched and distributed throughout the pancreas. MALDI Imaging MS of Neratinib for localization of drug distribution in mice tissue sections. **(A)** The dynamic range of the Neratinib signal (monoisotopic peak) in mice liver tissue sections after spotting neratinib standard with concentrations ranging from 0 to 500 pmol/µl is presented. **(B)** The single spectrum of the Neratinib standard spotted on a MALDI steel target shows the distinct isotopic pattern of the drug **(C)** compared to the simulated isotope pattern of $C_{30}H_{29}ClN_6O_3$ (Patiny and Borel, 2013). **(D)** This pattern could be unambiguously detected in the MALDI imaging MS study of pancreas tissue sections as shown by the representative single spectrum. **(E)** The neratinib signal at m/z 577.2 is clearly located in the treated pancreas sections (top) and absent in the non-treated control sections from obese db/db mice used in the study (bottom). **(F)** Distribution of Neratinib in the pancreas sections of wild-type mice 4h after injection (right) compared to the non-treated control (left). Scale bar, 100 µm

Publication II

Inhibition of PHLPP1/2 phosphatases rescues pancreatic β-cells in diabetes

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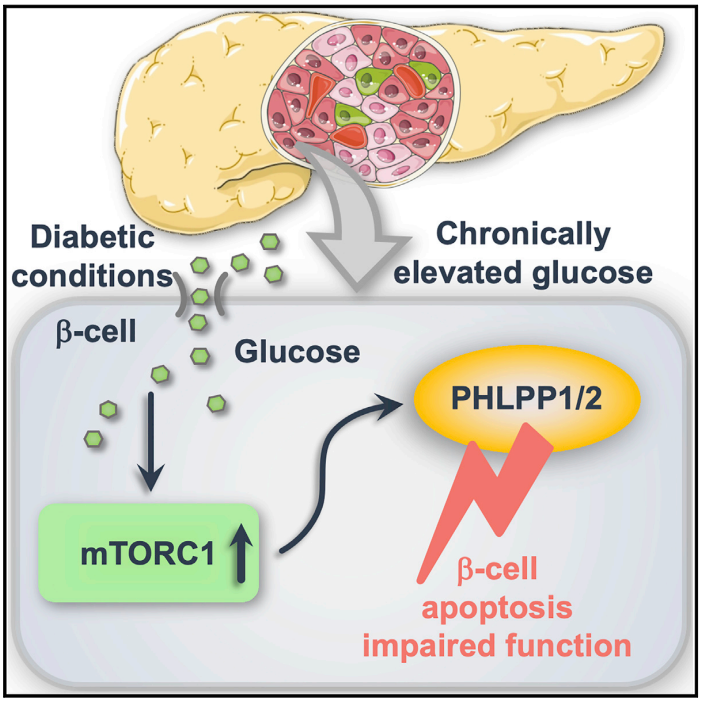
My contribution:
Designed, performed experiments, analysed data and assembled the figures for:
Figures: 1 A,(B – partially), E,F,H,I,(K, L – partially),M,N,O; 2 A,D,(E, F,G,H– partially); 3 (C,D,E – partially),F,G,H; 5 O,P,Q,R; 6 G,H; 7
Supplementary Figures: 1 B,C,D,E; 2 A,(B, C – partially),D,E,F; 3 D,E,F,G,H,I; 4 A,D; 6 B
Contributed in writing the paper.

Article

Cell Reports

Inhibition of PHLPP1/2 phosphatases rescues pancreatic β-cells in diabetes

Graphical abstract



Highlights

- PHLPP1/2 are highly elevated in metabolically stressed β cells in diabetes
- Metabolic-stress-induced mTORC1 hyper-activation leads to PHLPP upregulation
- PHLPPs regulate β-cell survival-dependent kinases AKT and MST1
- PHLPP inhibition restores glycemia, β-cell survival, and function

Authors

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In brief

Lupse et al. show that chronic metabolic stress and high sugar in diabetes leads to hyper-activation of the metabolism-control pathway mTORC1 and initiation of a triangle loop of cellular survival control by the phosphatase PHLPP. PHLPP inhibits pro-survival AKT, activates pro-apoptotic MST1, and ultimately leads to β-cell death and dysfunction.

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Inhibition of PHLPP1/2 phosphatases rescues pancreatic β-cells in diabetes

Blaz Lupse,[1] Karthika Annamalai,[1] Hazem Ibrahim,[1] Supreet Kaur,[1] Shirin Geravandi,[1] Bhavishya Sarma,[1] Anasua Pal,[1] Sushil Awal,[1] Arundhati Joshi,[1] Sahar Rafizadeh,[1] Murali Krishna Madduri,[1] Mona Khazaei,[1] Huan Liu,[1] Ting Yuan,[1] Wei He,[1] Kanaka Durga Devi Gorrepati,[1] Zahra Azizi,[1,2] Qi Qi,[3] Keqiang Ye,[3] Jose Oberholzer,[4] Kathrin Maedler,[1,5,6,*] and Amin Ardestani[1,2,5,*]

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SUMMARY

Pancreatic β-cell failure is the key pathogenic element of the complex metabolic deterioration in type 2 diabetes (T2D); its underlying pathomechanism is still elusive. Here, we identify pleckstrin homology domain leucine-rich repeat protein phosphatases 1 and 2 (PHLPP1/2) as phosphatases whose upregulation leads to β-cell failure in diabetes. PHLPP levels are highly elevated in metabolically stressed human and rodent diabetic β-cells. Sustained hyper-activation of mechanistic target of rapamycin complex 1 (mTORC1) is the primary mechanism of the PHLPP upregulation linking chronic metabolic stress to ultimate β-cell death. PHLPPs directly dephosphorylate and regulate activities of β-cell survival-dependent kinases AKT and MST1, constituting a regulatory triangle loop to control β-cell apoptosis. Genetic inhibition of PHLPPs markedly improves β-cell survival and function in experimental models of diabetes *in vitro*, *in vivo*, and in primary human T2D islets. Our study presents PHLPPs as targets for functional regenerative therapy of pancreatic β cells in diabetes.

INTRODUCTION

Type 2 diabetes (T2D) is a heterogeneous multifactorial metabolic disease, characterized by insulin resistance and progressive loss of functional β-cell mass. Pancreatic β-cell failure finally results from decreased insulin secretory function and/or β-cell death (Alejandro et al., 2015; Ashcroft and Rorsman, 2012; Butler et al., 2003; Weir et al., 2020), hallmarks of T2D; however, underlying molecular mechanisms are still not fully characterized, and there is currently no β-cell-specific therapy for a cure (Donath et al., 2019). In addition to β-cell death and dysfunction, other mechanisms, such as β-cell dedifferentiation (Cinti et al., 2016; Jeffery and Harries, 2016; Talchai et al., 2012) and failure of adaptive expansion because of impaired regeneration (Aguayo-Mazzucato and Bonner-Weir, 2018; Tiwari et al., 2016), have been proposed as possible causes for β-cell failure in T2D.

The coordinated cellular stress response and enormous metabolic adaptation are necessary for normal β-cell insulin-secretory function, glucose homeostasis, and prevention of T2D; these are largely directed by the highly complex dynamics of signal transduction pathways. Perturbations in β-cell signaling have complex consequences leading to imbalanced and improper transcriptional and post-transcriptional alterations, metabolic deterioration, continuous decline in β-cell function and viability and the cumulative development of diabetic complications. Thus, comprehensive understanding of cell-fate decisions during stress and metabolic overload will provide new targets for the development of therapeutic approaches aiming at prevention and repair of β-cell failure in T2D.

Serine-threonine phosphatases (STPs) are important components of multiple cell signaling nodes and serve as potential targets for drug development. The pleckstrin homology (PH) domain leucine-rich repeat protein phosphatases (PHLPPs) enzymes are members of the protein phosphatase 2C (PP2C) grouped in the protein phosphatase metal-dependent (PPM) family of STP (Brognard and Newton, 2008). The PHLPP family includes two isozymes, PHLPP1 (also referred to as suprachiasmatic nucleus circadian oscillatory protein [SCOP]) and PHLPP2 (Grzechnik and Newton, 2016). PHLPP1/2 are ubiquitously expressed and involved in several cellular processes, such as proliferation, survival, stress response, inflammation, memory formation, and T cell development (Brognard and Newton, 2008; Chen et al., 2013; Cohen Katsenelson et al., 2019; Gao

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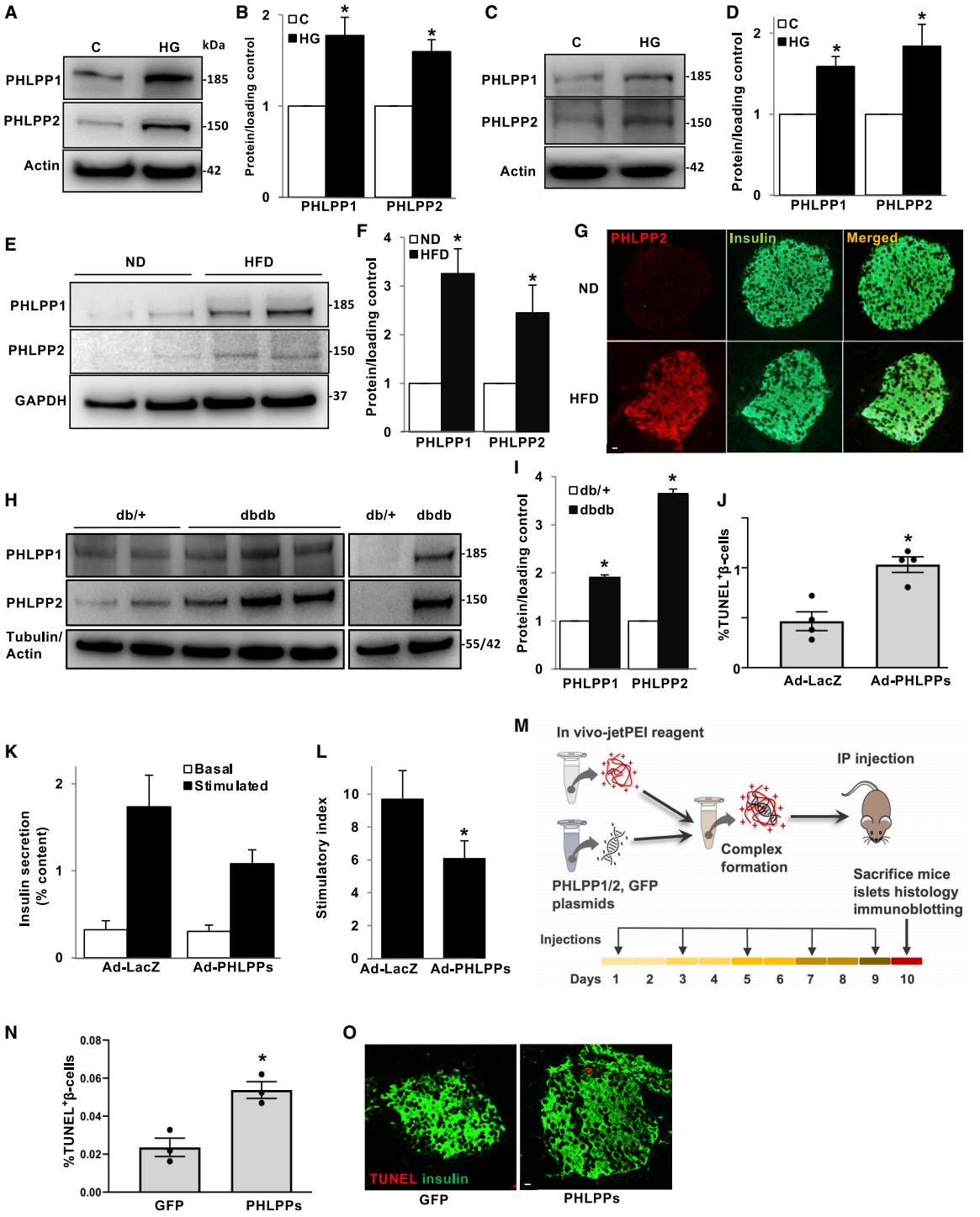

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et al., 2005; Liu et al., 2009; Masubuchi et al., 2010; Patterson et al., 2011). Initial studies identified PHLPP as a hydrophobic motif phosphatase to antagonize pro-survival signaling pathways. It is well-established that PHLPP1/2, when activated as a cellular response to cytotoxic stress, mediate cell death through dephosphorylation of multiple substrates, such as AKT, PKC, p70S6K, and MST1 (Gao et al., 2005, 2008; Liu et al., 2011b; Qiao et al., 2010). Notably, PHLPP1 single-nucleotide polymorphism (SNP) has been recently associated with T2D (Turki et al., 2013; Yako et al., 2016). In line with that genetic association, PHLPP1 expression is elevated in liver and skeletal muscle of insulin-resistant rodents (Behera et al., 2018; Liu et al., 2012) as well as in adipose tissue and skeletal muscle of human obese individuals, including patients with T2D (Andreozzi et al., 2011), indicating an important role for PHLPP1 in diabetes progression. Consistently, PHLPP1 is an important regulator of AKT signaling in the heart: knockdown or genetic deletion of PHLPP1 enhances pro-survival AKT activity in cardiac myocytes and, in turn, provides protection against ischemic injury (Aviv and Kirshenbaum, 2010; Chen et al., 2013; Miyamoto et al., 2010). Similarly, PHLPP1 depletion is neuroprotective and increases AKT signaling and survival in hippocampal and striatal neurons (Jackson et al., 2013).

So far, the physiological role of PHLPP1/2 in the human β-cell—whether PHLPP1/2 are upregulated in T2D, whether such upregulation would trigger β-cell death and impaired insulin secretion, and whether PHLPP1/2 inhibition can rescue β-cells in diabetes—is not known. In the present work, we aimed to investigate molecular and cellular mechanisms of PHLPP1/2-induced β-cell failure and to test whether inhibition of PHLPP1/2 prevent β-cell destruction and diabetes *in vivo*.

RESULTS

PHLPP1/2 are upregulated by diabetogenic conditions and impair β-cell survival and function

To identify PHLPP1/2 upregulation and its correlation with β-cell apoptosis, we exposed isolated human islets and the classically used rat β-cell line INS-1E to a diabetic milieu *in vitro* (chronically elevated glucose concentrations). PHLPP1/2 were highly upregulated at the protein level in INS-1E cells (Figures 1A and 1B) and in primary isolated human islets (Figures 1C and 1D). Consistently, PHLPP1/2 levels were increased in islets of hyperglycemic high fat/high sucrose (HFD)-fed mice for 16 weeks (Figures 1E and 1F). β-cell-specific PHLPP upregulation was confirmed by double staining for PHLPP2 and insulin from paraffin-embedded sections from HFD in comparison with normal diet (ND)-fed control mice (Figure 1G). Similarly, PHLPP1/2 protein levels were also elevated in islets of another model of T2D, the obese diabetic leptin-receptor-deficient *db/db* mice (Figures 1H and 1I). These data show that PHLPP1/2 are markedly elevated by pro-diabetic conditions *in vitro* and *in vivo* in mouse models of T2D.

To examine the role of PHLPP1/2 in β-cell death, we checked whether PHLPP1/2 overexpression alone is sufficient to promote β-cell apoptosis. PHLPP1/2 overexpression in INS-1E cells and human islets achieved by adenoviral gene transfer induced human (Figure 1J) and rodent (Figure S1A) β-cell apoptosis. In addition, overexpression of PHLPPs impaired glucose-stimulated insulin secretion (GSIS) in isolated human islets (Figures 1K and 1L) suggesting its detrimental role on both β-cell survival and insulin secretion.

To investigate whether PHLPPs directly induce β-cell death *in vivo*, we used the Polyplus-transfection reagent jetPEI, a polyethylenimine-based delivery system for safe and efficient introduction of nucleic acids into tissues *in vivo* as successfully reported previously for delivery into islets (Goyal et al., 2019; Kim et al., 2013). A solution of jetPEI carrier complexed with either HA-PHLPP1- and 2- or GFP (control)-expressing constructs was injected intraperitoneally (i.p.) into nondiabetic, wild-type (WT) mice. Mice were given a total of five injections every alternate day and sacrificed 1 day after the last injection (Figure 1M). PHLPP overexpression in islets was evaluated *ex vivo*. Immunohistochemistry of pancreatic sections as well as immunoblot analyses of isolated islets showed successful β-cell/islet upregulation of PHLPPs (Figures S1B and S1C). Chronic administration of PHLPPs significantly induced β-cell apoptosis, as compared with control GFP plasmids, represented by elevated TUNEL-positive β-cells (Figures 1N and 1O) as well as increased levels of caspase-3 cleavage (Figure S1C), a universal marker of apoptosis. In addition, our data show that there was no change in either proliferation or β-cell mass, suggesting that despite promoting apoptosis, a short time of 10-day PHLPP overexpression did not change β-cell mass (Figures S1D and

Figure 1. PHLPP1/2 is upregulated by diabetogenic conditions and impairs β-cell survival and function

(A–D) Representative western blots (A and C) and quantitative densitometry analysis (B and D) of INS-1E cells (A and B; n = 6) or isolated human islets (C and D; n = 6) treated with high glucose (22 mM) for 2 days.

(E and F) Representative western blots (E) and quantitative densitometry analysis (F) of isolated islets from normal diet (ND) or high-fat/high-sucrose diet (HFD)-fed mice for 16 weeks (n = 8).

(G) Representative images shown double immunostaining for PHLPP2 in red and insulin in green in pancreatic sections from ND- and HFD-treated mice.

(H and I) Representative western blots (H) and quantitative densitometry analysis (I) of isolated islets from 10-week-old diabetic *db/db* mice and their heterozygous *db/+* littermates (n = 5).

(J–L) Human islets transduced with LacZ control or PHLPP1 and PHLPP2 adenoviruses for 48 h. (J) Pooled TUNEL analysis (n = 4; an average of 18,718 β cells were counted from each treatment condition). (K) Insulin secretion during 1 h of incubation with 2.8 mM (basal) and 16.7 mM (stimulated) glucose, normalized to insulin content. (L) Insulin stimulatory index denotes the ratio of stimulated and basal (n = 5).

(M) Scheme of the *in vivo* experimental strategy. 8-week-old male C57BL/6 mice were intraperitoneally (i.p) administrated a mixture of *in vivo* jetPEI-PHLPP1/2 or -GFP control constructs, one of five injections on every alternate day, and sacrificed after 10 days, one day after the last injection.

(N and O) Pooled TUNEL analysis (N) and double staining for TUNEL (red) and insulin (green) (O) of isolated pancreases from GFP- or PHLPP1/2-transfected mice (n = 3; an average of 13,618 β cells were counted from each treatment condition).

Data are expressed as means ± SEM. *p < 0.05 compared with untreated or nondiabetic or LacZ or GFP control. White scale bars depict 10 μm.

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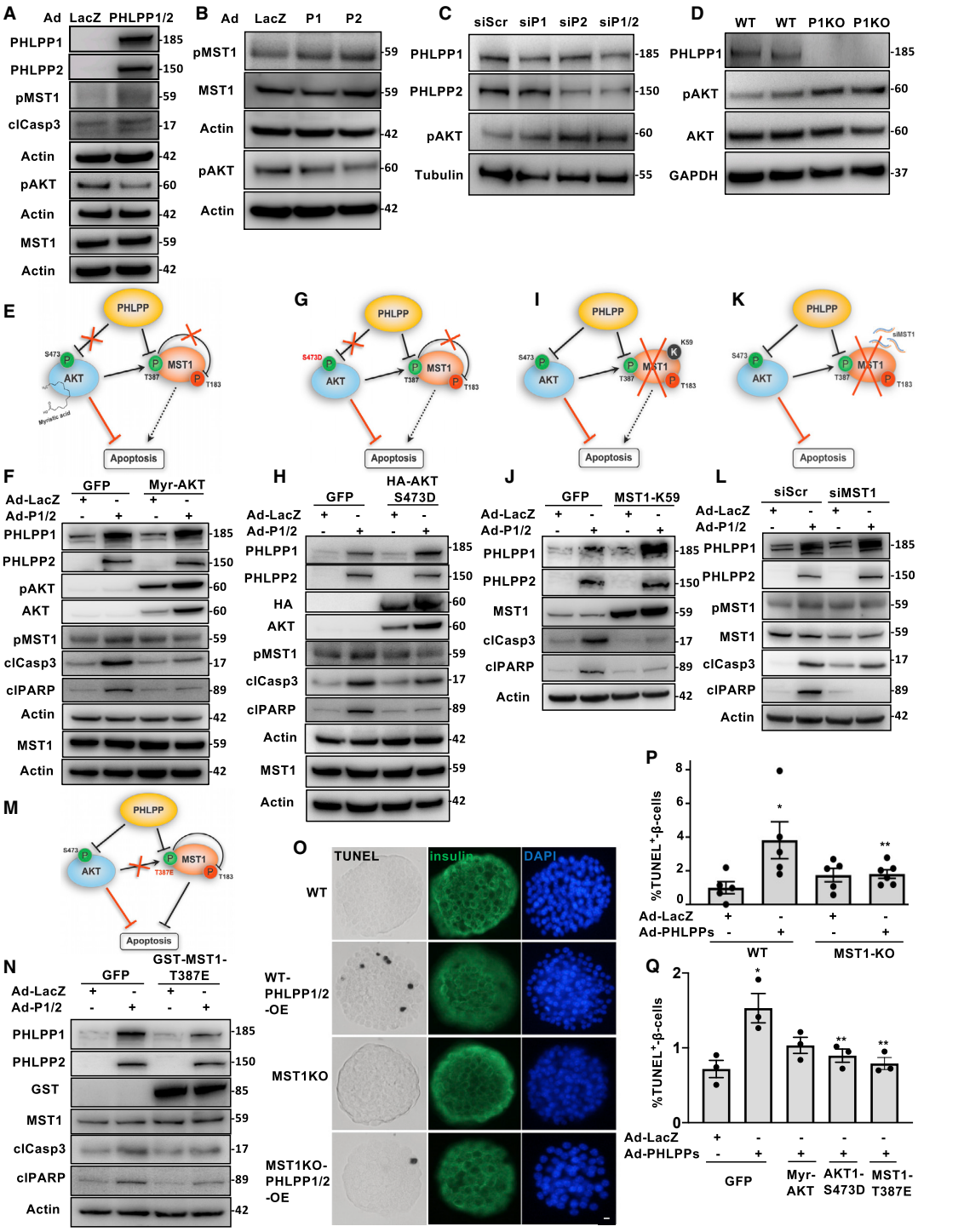

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S1E). Together, these data suggest that PHLPPs are highly elevated in diabetic β-cells and are harmful for β-cell survival.

PHLPP1/2 inhibit pro-survival AKT and activate pro-apoptotic MST1 signaling in pancreatic β-cells

To better understand the detrimental function of upregulated PHLPPs in β-cells, we analyzed the key down-stream substrates of PHLPPs. The first well established physiological substrate of PHLPP1/2 is AKT. PHLPP1 and PHLPP2 inactivate AKT through de-phosphorylation of AKT at Ser473 (Brognard et al., 2007; Gao et al., 2005). In light of the essential role of AKT in survival and adaptive growth of the pancreatic β cells (Elghazi and Bernal-Mizrachi, 2009; Yuan et al., 2018), we first sought to analyze the PHLPP-AKT axis that might coordinately control β-cell viability. PHLPP1/2 overexpression reduced AKT-Ser473 phosphorylation in human islets (Figure 2A) and in INS-1E cells (Figure 2B). Conversely, PHLPP1/2 knockdown in INS-1E cells enhanced phospho-AKT levels (Figure 2C), consistent with our observation in PHLPP1-deleted mouse embryonic fibroblasts (MEFs; Figure S2A) and isolated mouse islets (Figure 2D). Mitogens, such as insulin-like growth factor I (IGF-I) and insulin, exert their cell survival action primarily through phosphorylation and activation of AKT in the IRS-PI3K pathway (Trumper et al., 2000; Tuttle et al., 2001). In this line, we wondered whether modulation of PHLPP1/2 might alter insulin- or IGF-I-induced AKT phosphorylation by direct functional regulation of AKT. Indeed, ectopic overexpression of PHLPP1/2 alone or together diminished stimulated AKT phosphorylation in β cells (Figures S2B and S2C). Similar changes occurred *in vivo*; pancreatic islets isolated from regularly fed *in vivo* jetPEI-PHLPP1/2-transfected mice showed a decrease in AKT-Ser473 phosphorylation (Figure S2D).

The second important PHLPP1/2 target is mammalian sterile 20-like kinase 1 (MST1); PHLPP1/2 directly bind and activate pro-apoptotic MST1 signaling by dephosphorylation at the auto-inhibitory MST1-Thr-387 site (Qiao et al., 2010). Because MST1 is a key regulator of β-cell survival in diabetes (Ardestani et al., 2014), we next investigated the potential upstream role of PHLPPs on MST1 regulation of β-cell apoptosis. PHLPP1/2 overexpression activated MST1 as manifested by increased phosphorylation at the MST1-Thr183-activating residue in human islets (Figure 2A) and INS-1E cells (Figure 2B). Because the PHLPP substrates AKT and MST1 mutually inhibit each other (Ardestani et al., 2014), several complementary experimental settings were designed to investigate the PHLPP-AKT-MST1 crosstalk in β cells in depth:

(1) We started with overexpression of Myr-AKT1, a constitutively active form of AKT with a myristoylation sequence attached to the membrane, which is not sensitive to PHLPPs (Figure 2E). Myr-AKT1 counteracted PHLPP-induced MST1 activation and caspase-3 and PARP cleavage (apoptosis readouts) in β cells (Figure 2F), suggesting that the lack of PHLPP-induced AKT inhibition antagonizes MST1 activation and apoptosis.
(2) In the second experiment, INS-1E cells were transfected with the phospho-mimetic mutant AKT1-S473D, in which serine 473 is permanently replaced with the phospho-mimetic amino acid aspartic acid. Thus, AKT can no longer be dephosphorylated by PHLPP and is constitutively active (Figure 2G). AKT1-S473D mutant suppressed MST1 activation and β-cell apoptosis triggered by PHLPP overexpression (Figure 2H), indicating the critical role of AKT-Ser473 phosphorylation in regulating AKT as well as MST1 activity and subsequent survival.
(3) In the third experiment, the kinase-dead mutant of MST1 (K59R-MST1; Figure 2I) was overexpressed. The critical lysine in the ATP binding pocket of MST1 kinase is mutated so that it cannot receive any ATP and, thus, is inactive (Yamamoto et al., 2003). K59R-MST1 antagonized the pro-apoptotic effect of PHLPPs overexpression in β cells as shown by reduced caspase-3 and PARP cleavage (Figure 2J).
(4) To further confirm whether MST1 hyperactivity has a role in β-cell apoptosis upon PHLPPs overexpression, small-interfering RNA (siRNA) was used to suppress MST1 expression in INS-1E cells (Figure 2K). Consistent with the dominant-negative results described in Figure 2J, MST1 silencing abrogated PHLPP-induced β-cell apoptosis, especially seen by the fully diminished PARP cleavage (Figure 2L).
(5) Given that PHLPP-mediated dephosphorylation of MST1 at the Thr387 inhibitory site increases the activity of MST1 (Qiao et al., 2010), we examined whether MST1-Thr387 phosphorylation mediates the PHLPP-dependent regulation of MST1 in β-cells. INS-1E cells were transfected with the phospho-mimetic mutant MST1-T387E, in which

Figure 2. PHLPP1/2 inhibits pro-survival AKT and activates pro-apoptotic MST1 signaling in pancreatic β-cells

(A–D) Representative western blots of isolated human islets (A) and INS-1E cells (B) transduced with LacZ control or PHLPP1 and PHLPP2 adenoviruses for 48 h or (C) transfected with PHLPP1 and/or PHLPP2 siRNA or control siScr for 2 days. (D) Representative western blots of islets isolated from WT and PHLPP1-KO mice (A–D: n = 3).

(E–N) Schematic cartoons and representative western blots of INS-1E cells overexpressed with adenoviruses for LacZ (control) or PHLPP1/2 and transfected with GFP or siScr (control), Myr-AKT1 (E and F), HA-tagged AKT-S473D (G and H), kinase dead MST1-K59R (I and J), siRNA to MST1 (K and L), or MST1-T387E (M and N) plasmids (all n = 2).

(O and P) Representative images of triple staining for TUNEL (black), insulin (green) and DAPI (blue) (O; scale bar depicts 10 μm) and pooled TUNEL analysis (P) of isolated islets from MST1-KO mice and their WT littermates after transduction with adenoviruses for LacZ (control) or PHLPP1/2 (n = 5–6; an average of 15,301 β cells were counted from each treatment condition).

(Q) Pooled TUNEL analysis of isolated human islets overexpressed with adenoviruses for LacZ (control) or PHLPP1/2 and transfected with GFP (control) or Myr-AKT1 or HA-tagged AKT-S473D, or MST1-T387E plasmids (n = 3; an average of 14,034 β cells were counted from each treatment condition).

Data are expressed as means ± SEM. *p < 0.05 compared with LacZ control. **p < 0.05 MST1-KO-PHLPP or PHLPP-AKT-S473D or PHLPP- MST1-T387E compared with WT-PHLPP or PHLPP-GFP.

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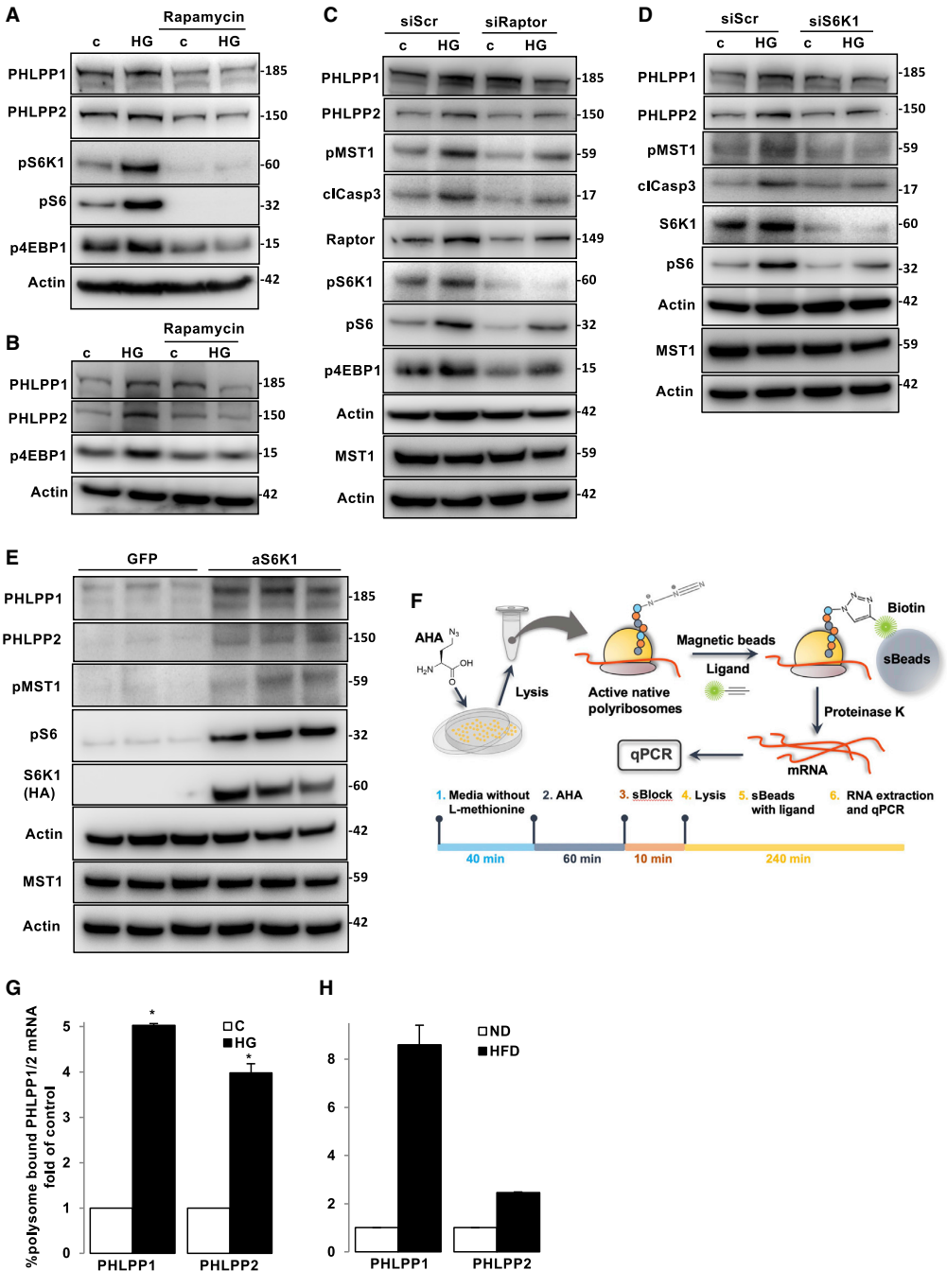

Figure 3. mTORC1 hyper-activation induces PHLPPs translation

(A and B) Representative western blots of INS-1E cells (A) and isolated human islets (B) pre-treated with 100 nM rapamycin and cultured with 22.2 mM glucose for 2 (INS-1E) and 3 (human islets) days. n = 3.

(C and D) Representative western blots of INS-1E cells transfected with siS6K1 (C), siRaptor (D) or siScr and then exposed to 22.2 mM glucose for 2 days. n = 3

(E) Representative western blots of INS-1E cells transfected with active S6K1 or GFP control plasmids for 2 days. n = 3.

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threonine 387 is permanently replaced by the phosphomimetic amino acid glutamic acid. Thus, MST1 is no longer dephosphorylated by PHLPP and MST1-T387E mimics the inhibitory phosphorylation of MST1 rendering the protein inactive (Figure 2M). Overexpression of the MST1-T387E mutant reduced PHLPP-induced β-cell apoptosis (Figure 2N), showing the important role of MST1-T387 dephosphorylation by PHLPP in the regulation of MST1 activation and β-cell death.

(6) To further support the role of MST1 as the main mediator of PHLPP-induced apoptosis in β-cells, we isolated islets from MST1 knockout (MST1-KO) or WT littermate mice and found that MST1-KO islets were largely resistant to PHLPP1/2-induced apoptosis (Figures 2O, 2P, and S2E). We also tested whether decreased AKT or increased MST1 activities were responsible for PHLPP-induced β-cell death in human islets by direct introduction of PHLPP-insensitive AKT and MST1 mutants. Both AKT/MST1 mutants significantly abolished the number of TUNEL-positive apoptotic human β-cells upon PHLPPs overexpression confirming previous observations in rodent β-cells (Figures 2Q and S2F).

In summary, multiple gain- and loss-of-function experimental approaches targeting endogenous AKT and MST1 activities as well as PHLPP site-specific mutation analysis using phospho-mimetic mutants of AKT (AKT-S473D) and MST1 (MST1-T387E) showed that the critical kinases AKT and MST1 regulate the pro-apoptotic action of PHLPPs in β cells.

mTORC1 hyper-activation induces PHLPPs translation

Chronic exposure of INS-1E cells as well as human islets to elevated glucose concentrations upregulated PHLPPs levels (Figure 1) without changing PHLPP1/2 mRNA expression (Figures S3A and S3B) as well as PHLPP1/2 protein stability (Figure S3C), suggesting that high-glucose-induced PHLPP1/2 induction neither occurred at transcriptional nor post-translational levels. The mechanistic target of rapamycin complex 1 (mTORC1) signaling is a principal regulator of protein translation to control major cellular functions, such as metabolism, growth, and survival (Saxton and Sabatini, 2017). We have previously reported aberrant mTORC1 hyper-activation in diabetic islets (Yuan et al., 2017). To define whether mTORC1 regulates PHLPPs expression, mTORC1 signaling was inhibited by chemical inhibitors against mTORC1 (rapamycin) and S6K1, a major down-stream effector of mTORC1 (PF-4708671; S6K1i) (Pearce et al., 2010). Activation of mTORC1 was demonstrated by increased phosphorylation of its downstream target S6K1 at Thr389 (pS6K), and the direct S6K substrate ribosomal protein S6 at Ser235/236 (pS6), as well as eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) at Thr37/46 (p4E-BP1). In TSC2-KO MEFs, an experimental model of constitutive mTORC1 activation (Bachar et al., 2009), PHLPP1/2 were highly upregulated compared with WT MEFs, and blocking mTOR signaling by rapamycin resulted in a marked decrease of PHLPP1/2 protein expression in TSC2-KO, but not in WT, MEFs (Figure S3D). Likewise, in pancreatic β cells under glucotoxic conditions, mTORC1 inhibition by rapamycin resulted in decreased levels of pS6K1, pS6, and p4EBP1 (mTORC1 readouts) and blocked high-glucose-induced PHLPP1 and PHLPP2 upregulation in INS-1E cells (Figure 3A) and in isolated human islets (Figure 3B) providing direct evidence of the PHLPP regulation by mTORC1, which was upregulated upon chronic exposure to increased glucose concentrations (Figures 3A and 3B). Consistently, pharmacological inhibition of S6K1 by PF-4708671 suppressed high-glucose-induced PHLPPs induction in β cells (Figure S3E). In line with that observation, selective inhibition of endogenous mTORC1 by siRNA-mediated silencing of raptor, mTORC1's critical subunit, counteracted mTORC1 signaling, reduced PHLPPs levels, and substantially protected INS-1E cells from high-glucose-induced MST1 activation and apoptosis (Figure 3C). Likewise, knockdown of S6K1 demonstrated that the depletion of mTORC1 down-stream signaling markedly reduced PHLPPs as well as MST1 activation and apoptosis (Figure 3D). This further corroborated hyper-activated mTORC1 as an up-stream regulator of PHLPP1/2 expression in the context of glucose-induced β-cell apoptosis. To further explore whether mTORC1-S6K signaling is a principal regulator of PHLPPs in β cells, we overexpressed the constitutively active form of S6K1 in INS-1E cells. Notably, sustained S6K1 overexpression effectively induced PHLPP1/2; this was accompanied by higher MST1 phosphorylation, recapitulating hallmarks of metabolically stressed β cells cultured under diabetes-associated glucotoxic conditions, including induction of PHLPP1/2 as well as activating MST1 (Figure 3E). Likewise, pharmacological induction of mTORC1 by the small-molecule mTOR activator 3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO) (Ge et al., 2014; Peng et al., 2014) or MHY1485 (Choi et al., 2012) upregulated PHLPPs, activated pro-apoptotic MST1, and impaired β-cell survival in INS1-E β-cells (Figures S3F–S3I).

An elegant previous study used high-resolution transcriptome-scale ribosome profiling to show that subsets of mRNAs that are specifically regulated by mTORC1 at the translational level consist of established 5′ terminal oligopyrimidine (TOP) or previously unrecognized TOP-like motifs (Thoreen et al., 2012). Interestingly, the 5′ untranslated region (UTR) of PHLPP1 mRNA contains a TOP motif (5′-CTTCTCCCTTCTCC-3′) and PHLPP2 mRNA contains a TOP-like motif (5′-CCTTGCC-3′), proposing potential mTORC1-dependent regulation of PHLPPs at the translational level (Liu et al., 2011a; Wen et al., 2013). Because mTORC1 induces PHLPPs, we hypothesized that the expression of PHLPP1/2 under diabetic conditions is upregulated at the translational level. We have used AHARIBO, a

(F) Experimental strategy of the AHARIBO-based isolation of active polyribosomes and associated RNAs.
(G and H) qPCR measurement of PHLPP1 or PHLPP2 mRNA associated with polysomes of INS-1E cells treated with high glucose (G) (n = 3) or of isolated islets from mice fed for 16 weeks with a ND or HFD (H) (n = 2 independent experiments; each pooled from 8–10 mice/condition).
Data are expressed as means ± SEM. *p < 0.05 compared with untreated controls.

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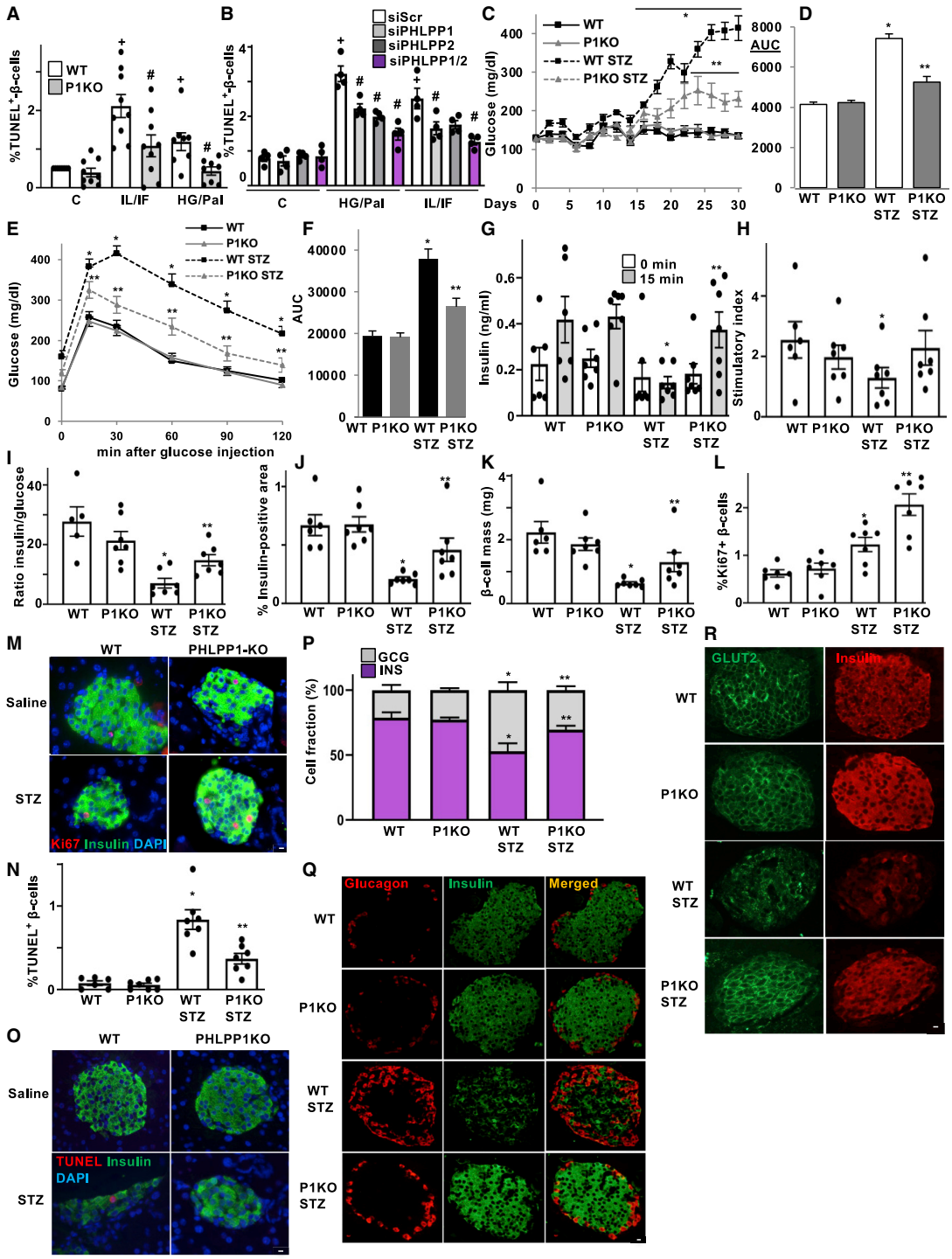

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minimally invasive, non-canonical amino acid tagging and isolation method of active polyribosomes (RiboMINATI). The protocol relies on the pulse incubation of cell cultures with L-azidohomoalanine (AHA). Then, a small molecule (sBlock) blocks the nascent peptide attached to the ribosome. A biotin tag is linked to the newly synthesized AHA-labeled proteins, and mRNAs associated to polysomes are analyzed by qPCR (Figure 3F). High-glucose-treated INS-1E cells (Figure 3G) and islets isolated from long-term HFD-fed diabetic mice (Figure 3H) displayed a higher percentage of PHLPP1/2 mRNAs bound to polysomes as compared with WT islets, suggesting a marked elevation in PHLPPs translation.

Altogether, these results indicate that PHLPP levels under diabetic conditions are regulated by mTORC1 at the translational level.

Loss of PHLPPs attenuated stress-induced β-cell injury *in vitro* and *in vivo*

To examine whether PHLPP1/2 upregulation is causative for β-cell apoptosis, islets isolated from WT and PHLPP1-KO mice were chronically exposed to glucolipotoxic conditions as well as the mixture of pro-inflammatory cytokines interleukin-1beta (IL-1β) and interferon gamma (IFN-γ). PHLPP1 deletion potently inhibited pro-inflammatory cytokine- as well as high-glucose/palmitate-induced β-cell death (Figure 4A). In addition, in human islets transfected with siRNAs directed to PHLPP1 and/or PHLPP2 before exposure to pro-diabetic stimuli (Figure S4A), apoptosis triggered by pro-inflammatory cytokines as well as by the mixture of high-glucose/palmitate was significantly abolished by the knockdown of PHLPP1 or PHLPP2 individually or together (Figure 4B).

Because PHLPP1/2 silencing improved islet β-cell survival under diabetic conditions *in vitro*, we hypothesized that PHLPP1 deficiency might be beneficial against β-cell injury and diabetes development *in vivo*. PHLPP1-KO mice are viable, fertile, and showed no significant differences in basal glycemia, food intake, and body weight compared to WT control mice (Figures 5, S5A, and S5B). We tested whether PHLPP1-KO mice are protected from diabetes progression in the multiple low-dose streptozotocin (MLD-STZ) model of β-cell destruction and diabetes (Horwitz et al., 2018; Luo et al., 2019). MLD-STZ for 5 consecutive days induced progressive hyperglycemia and glucose intolerance rendering WT mice overtly diabetic, whereas blood glucose in PHLPP1-KO mice was robustly attenuated (Figures 4C and 4D), and glucose tolerance significantly improved at all time points (Figures 4E and 4F). In line with the impairment in glucose tolerance, glucose-induced insulin secretion was fully blunted in the MLD-STZ-treated WT mice, whereas PHLPP1-KO animals exhibited a marked restoration in insulin secretion 15 min after the glucose challenge; stimulatory index was unchanged compared with non-STZ injected mice, together with a significantly increased insulin-to-glucose ratio in PHLPP1-KO mice, compared with STZ-injected WT controls (Figures 4G–4I).

Consistent with the metabolic improvements, β-cell volume and β-cell mass were significantly restored in PHLPP1-KO, compared with STZ-WT mice (Figures 4J and 4K). To determine whether the regeneration of the β-cell mass was a result of an increased β-cell number because of augmented β-cell replication and/or decreased β-cell apoptosis, we further assessed the effects of PHLPP1 ablation on β-cell survival and proliferation. Together with increased β-cell apoptosis in WT-STZ animals, β-cell proliferation—as represented by double-labeled Ki67/insulin-positive β-cells—was elevated, showing an enhanced compensatory capacity in response to STZ-induced β-cell injury (Figures 4L–4O). PHLPP1 deletion fostered β-cell proliferation (Figures 4L and M) and suppressed β-cell apoptosis (Figures 4N and 4O), compared with the STZ-WT group. Loss of PHLPP1 had no effect on basal β-cell mass and turnover (apoptosis/proliferation) in non-diabetic mice. These results suggest that PHLPP1 ablation restores β-cell mass predominantly as a result of reduced β-cell apoptosis and a demand for β-cell compensation. Islet cells from MLD-STZ-treated WT mice were architecturally disrupted, with fewer insulin-positive β cells and proportionally more glucagon-positive α cells compared with that of untreated WT mice (Figures 4P and 4Q). In contrast, the percentage of glucagon-positive α cells as well as insulin-positive β cells in MLD-STZ-injected PHLPP1-KO islets was similar to non-STZ-treated WT control mice and confined to the rim of the islets (Figures 4P and 4Q).

We next checked whether PHLPP1 deficiency could also restore the expression of several key markers of the glucose-sensing machinery as well as of insulin production. Immunostaining of pancreatic sections from STZ-treated mice showed a profound loss in both nuclear PDX1- and NKX6.1-positive cells. Many cells within the islets, which still express insulin, had lost their PDX1 or NKX6.1 expression. Such PDX1/NKX6.1 protein

Figure 4. Loss of PHLPPs attenuated stress-induced β-cell injury *in vitro* and *in vivo*

(A and B) TUNEL analysis of isolated islets from PHLPP1-KO mice and their WT littermates (A) and of isolated human islets transfected with PHLPP1 and/or PHLPP2 siRNA or control siScr (B) and then treated with 22.2 mM glucose plus 0.5 mM palmitate (HG/Pal) or the mixture of 2 ng/mL IL1β plus 1,000 U/mL IFN-γ (IL/IF) for 3 days. n = 4–9.

(C–R) PHLPP1-KO and WT control mice injected with streptozotocin (STZ; 40 mg per kg body weight) or saline for 5 consecutive days (n = 6–7). (C) Random-fed blood glucose measurements after first saline or STZ injection (day 0) over 30 days and (D) respective area-under-the curve (AUC) analyses. (E) i.p. glucose tolerance test (GTT) and (F) respective AUC analyses in PHLPP1-KO and WT mice. (G) Insulin levels during an i.p. GTT measured before (0 min) and 15 min after glucose injection and expressed (H) as the ratio of secreted insulin at 15 to 0 min (stimulatory index). (I) Ratio of secreted insulin and glucose calculated at the fed state. (J) Insulin-positive area and (K) β-cell mass (given as the percentage of insulin-positive to the entire pancreatic section area from 10 sections spanning the width of the pancreas). (L–O) Quantitative analyses and representative images from triple staining for Ki67 (L and M; an average of 11,609 β cells were counted from each treatment condition) or TUNEL (N and O; an average of 12,733 β cells were counted from each treatment condition), insulin, and DAPI; expressed as the percentage of TUNEL- or Ki67-positive β cells ± SEM (P and Q) Quantitative analyses (P) and representative images (Q) of the percentage of α cells (red) and β cells (green). (R) Representative double-staining for Glut2 (green), and insulin (red).

Data are expressed as means ± SEM. $^{+}p < 0.05$ versus untreated control. $^{\#}p < 0.05$ PHLPP1-KO or siPHLPP1/2 versus WT or siScr at the same treatment conditions. $^{*}p < 0.05$ WT-STZ compared with WT saline-injected mice, $^{**}p < 0.05$ PHLPP1-KO-STZ versus WT-STZ mice. White scale bars depict 10 μm.

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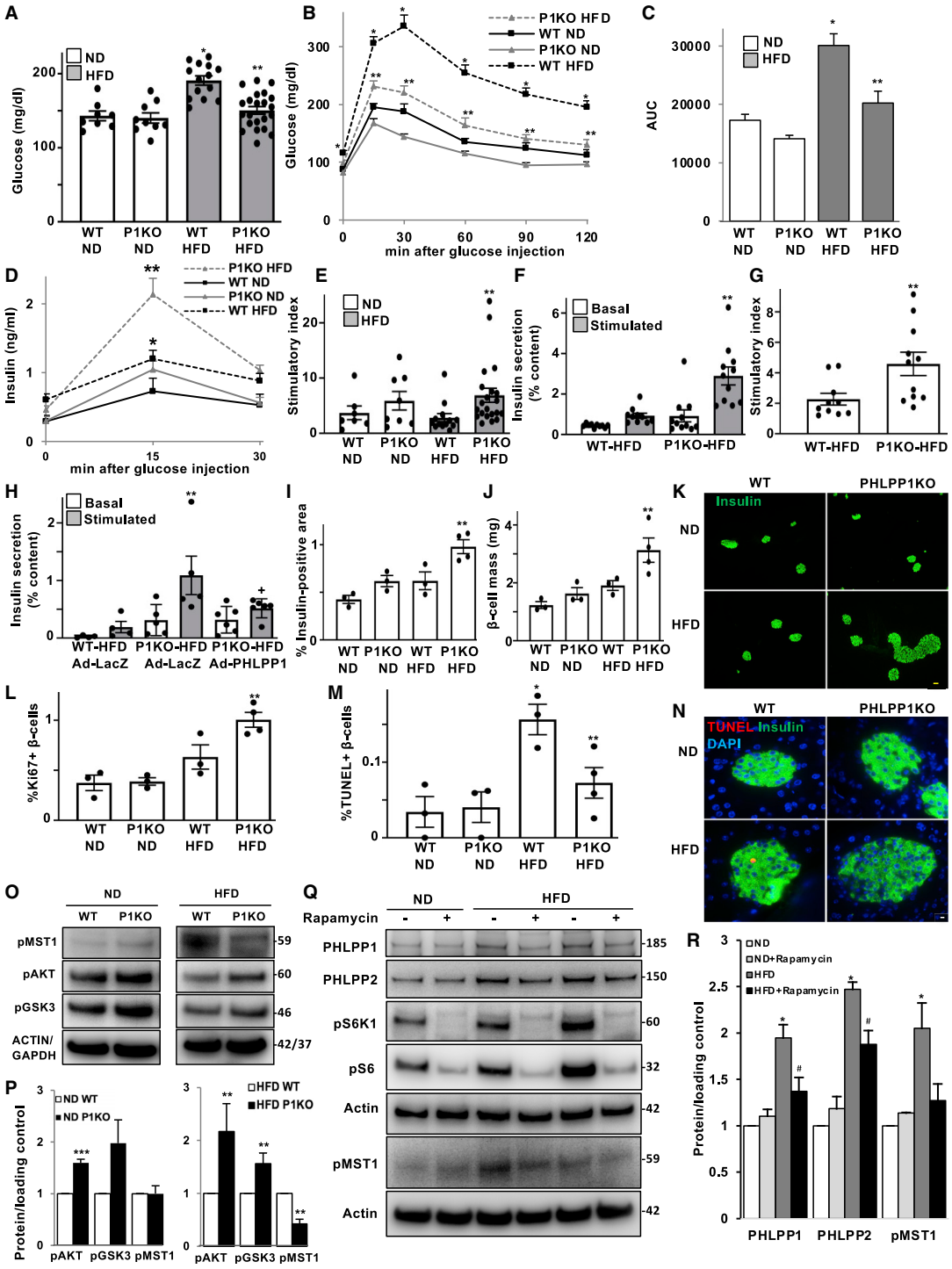

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expression was markedly restored by PHLPP1 inhibition (Figures S4B and S4C). Consistently, although the expression of the PDX1 canonical down-stream target GLUT2 was reduced and its membrane localization disrupted in β cells of MLD-STZ-treated WT mice, GLUT2 expression as well as its membrane localization was largely preserved in MLD-STZ-treated PHLPP1-KO islets, compared with that of STZ-injected WT mice (Figure 4R). This was also confirmed *in vitro* in isolated mouse islets, in which PHLPP1 deletion restored the STZ-induced loss of GLUT2 expression (Figure S4D).

The combination of these metabolic and morphological data suggests that PHLPP1 ablation leads to enhanced β-cell mass and proliferation, reduced apoptosis, and restored insulin secretion and glucose tolerance in an *in vivo* model of β-cell destruction and diabetes.

PHLPP1 deletion protects from HFD-induced diabetes

To further characterize the physiological relevance of our findings in a second, diet-induced diabetes model, we sought to examine whether PHLPP1 is indispensable for the long-term β-cell compensatory response in the diet-induced obesity mouse model of HFD-induced diabetes. For this purpose, WT and PHLPP1-KO male mice were placed on either ND or HFD for 17 weeks, which led to chronic hyperglycemia, insulin resistance, and β-cell failure in WT mice (Ardestani et al., 2014; Collins et al., 2010). On an ND diet, PHLPP1-KO mice were normal, healthy, and indistinguishable from WT controls; systemic PHLPP1 deletion had no effect on weight gain or on food intake in either ND or HFD groups (Figures S5A and S5B). When maintained on a long-term HFD, WT mice developed mild hyperglycemia and drastic impairment in glucose tolerance, which was robustly attenuated in HFD-treated PHLPP1-KO mice (Figures 5A–5C). To assess whether these metabolic improvements were due to changes in insulin sensitivity, we performed an insulin tolerance test. Under the non-diabetogenic conditions of a chow diet, WT and PHLPP1-KO mice had a similar response to exogenous insulin. Under HFD conditions, PHLPP1-KO mice had a slightly better insulin tolerance compared with that of insulin-resistant WT mice (Figure S5C). However, this is unlikely to be the major mechanism for the robust systemic improvement of glucose tolerance in obese HFD-treated PHLPP1-KO mice. PHLPP1 deletion significantly enhanced insulin secretion as well as stimulatory index during an i.p. glucose challenge in HFD-fed mice (Figures 5D and 5E). To further assess whether the improvements in glucose homeostasis in PHLPP1-KO mice were directly linked to insulin secretion, we measured GSIS *ex vivo* in isolated islets from HFD-fed groups. Islets from PHLPP1-KO mice on an HFD exhibited substantially increased insulin secretion in response to glucose; also, the stimulatory index was highly improved compared with that of WT-HFD mice (Figures 5F and 5G), recapitulating the *in vivo* phenotype. We next asked a critical question: does reconstitution of the PHLPP1 reverse the enhancement in glucose responsiveness evident in HFD-treated PHLPP1-KO islets? PHLPP1 overexpression resulted in a reversal in GSIS in PHLPP1-reconstituted islets isolated from HFD-PHLPP1-KO mice compared with LacZ-transduced HFD-PHLPP1-KO counterparts suggesting a potential cell-autonomous action of PHLPP1 in the regulation of insulin secretion (Figure 5H).

Consistent with the improved insulin secretion *in vivo* and *ex vivo*, PHLPP1-KO mice displayed a greater compensatory response, i.e., β-cell volume and β-cell mass were significantly increased relative to WT control mice under the HFD diet (Figures 5I–5K). These findings implicate functional β-cell-mass restoration as a key factor for the metabolic benefits in the PHLPP1-KO mice. Similar to the STZ model of β-cell destruction (Figure 4), PHLPP1-KO mice showed significantly more β-cell proliferation (Figure 5L) and fewer β-cell apoptosis (Figures 5M and 5N), compared with that of WT-HFD mice.

In addition, western blot analysis of isolated islets from ND-treated WT and PHLPP1-KO mice showed PHLPP deletion increased phosphorylation of AKT and its downstream target GSK3, whereas MST1 phosphorylation remained unchanged (Figures 5O and 5P). Importantly, genetic inhibition of PHLPP1 resulted in suppressed MST1 activation and restored AKT activation in islets isolated from HFD-subjected mice (Figures 5O and

Figure 5. PHLPP1 deletion protects from HFD-induced diabetes

(A–C) PHLPP1-KO and WT control mice were fed an ND or an HFD (“Surwit”) for 17 weeks. (A) Random-fed blood glucose, (B) i.p. GTT, and (C) respective AUC analyses. n = 8–22.

(D and E) Insulin secretion during an i.p. GTT measured before (0 min), 15 and 30 min after glucose injection and expressed as (E) the ratio of secreted insulin at 15 to 0 min (stimulatory index) (n = 7–21).

(F and G) Islets were isolated from all four treatment groups, cultured overnight, and subjected to an *in vitro* GSIS (F). Insulin secretion during 1 h of incubation with 2.8 mM (basal) and 16.7 mM glucose (stimulated), normalized to insulin content, and (G) the stimulatory index denotes the ratio of stimulated to basal insulin secretion (n = 10–11).

(H) Islets isolated from HFD-fed groups transduced with LacZ control or PHLPP1 adenoviruses for 1 day and subjected to an *in vitro* GSIS (n = 4-6).

(I–K) Insulin-positive area (I) and β-cell mass (J) (given as percentage of the insulin-positive area to the entire pancreatic section from 10 sections spanning the width of the pancreas) and (K) respective representative images (n = 3–4; yellow scale bar depicts 50 μm).

(L–N) Quantitative analyses from double/triple staining for Ki67 (L) or TUNEL (M) (and representative images: N; white scale bar scale depicts 10 μm), insulin, and DAPI expressed as percentage of Ki67- or TUNEL-positive β cells (n = 3–4; an average of 7,648 [Ki67] or 9,009 [TUNEL] β cells were counted from each treatment condition).

(O and P) Representative western blots (O) and quantitative densitometry analysis (P) of isolated islets from WT and PHLPP1-KO mice fed an ND or an HFD (n = 3–4). Islet samples isolated from mice under ND or HFD were run on different gels. For each cohort, protein expression signal is normalized to the corresponding WT mice and quantitative densitometry analysis as a fold of the change is presented separately.

(Q and R) Representative western blots (Q) and quantitative densitometry analysis (R) of islets isolated from ND- and HFD-fed mice treated with 100 nM rapamycin (PHLPP1/PHLPP2, n = 7; pMST1, n = 3).

Data are expressed as means ± SEM. *$p < 0.05$ WT-HFD versus WT-ND mice, **$p < 0.05$ PHLPP1-KO-HFD versus WT-HFD mice, ***$p < 0.05$ PHLPP1-KO-ND versus WT-ND, $^{+}p < 0.05$ HFD-PHLPP1-KO-AdPHLPP1 versus HFD-PHLPP1-KO-LacZ, $^{\#}p < 0.05$ rapamycin-HFD versus control-HFD.

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5P), confirming the regulatory MST1-AKT axis downstream of PHLPPs *in vivo* in diabetic islets. Because mTORC1 induced PHLPPs *in vitro*, we also investigated whether diabetes-induced PHLPP expression is regulated by mTORC1 in ND- or HFD-treated mouse islets. Similar to INS-1E cells and human islets cultured under high-glucose conditions, mTORC1 inhibition by rapamycin reduced the levels of both PHLPP isoforms in isolated islets from HFD-treated diabetic mice (Figures 5Q and 5R), further confirming mTORC1 signaling as an upstream regulator of PHLPPs. In addition, rapamycin did not significantly affect the PHLPP levels under ND control conditions (Figures 5Q and 5R).

Altogether, PHLPP1 genetic inhibition elicited a robust glucose-lowering response in obese hyperglycemic mice through—at least, in part—a coordinated increase in both β-cell mass and secretory function.

Genetic inhibition of PHLPP1/2 improves insulin secretion and β-cell survival in human islets from patients with T2D

To identify whether the observed PHLPP1/2 upregulation in research models of diabetes is relevant to human T2D and may contribute to its pathogenesis, we investigated islets from patients with T2D. Western blot analysis of human islets from patients with T2D exhibited significant upregulation of PHLPP1/2, compared with islets from non-diabetic individuals (Figures 6A and 6B). In line with the pattern seen under a pro-diabetic milieu *in vitro*, PHLPP1 and PHLPP2 mRNA abundance was unchanged in T2D human islets (Figure S6A), again indicating that a post-transcriptional mechanism was responsible for the increase in PHLPPs. Immunofluorescence staining confirmed increased protein expression of PHLPP2 in the insulin-positive area in islets from paraffin-embedded pancreas sections from autopsies of patients with T2D, in comparison to an only faint PHLPP2 expression in non-diabetic controls (Figure 6C). Increased β-cell apoptosis as a major pathological feature of diabetes was observed in human islets under the same experimental settings (Dharmadhikari et al., 2017). To understand the patho-physiological effect of increased PHLPPs in human T2D islets, siRNA-mediated knockdown was used to examine whether PHLPP inhibition restores β-cell function and survival (Figure S6B). Importantly, although genetic inhibition of PHLPP1 or PHLPP2 alone or together had no effect on insulin secretion in the nondiabetic cohort, their loss significantly improved GSIS in five independent human islet preparations isolated from organ donors with T2D (Figures 6D and 6E). In addition, β-cell viability was restored by genetic inhibition of PHLPPs in T2D islets (Figure 6F). Moreover, targeted inhibition of endogenous mTORC1 by siRNA-mediated silencing of raptor in isolated T2D islets substantially decreased PHLPPs (Figures 6G and 6H), further indicating the mTORC1-dependent PHLPPs upregulation in human T2D islets, in confirmation with the PHLPP reduction seen by mTORC1 inhibition in β cells and human islets under long-term high-glucose treatment (Figure 3). This shows a detrimental effect of abnormally upregulated PHLPPs on β-cell function and survival in human T2D islets and suggests that the higher protein expression of PHLPPs might be linked to the impaired insulin secretion and metabolic deterioration in human diabetes (Figure 7).

DISCUSSION

In the present study, we provide direct evidence for PHLPP protein upregulation in β-cells as an initiator path toward β-cell failure in diabetes, because (1) PHLPPs were highly upregulated in human islets and β-cells under glucotoxic conditions *in vitro* and in islets from diabetic mouse models and in patients with T2D; (2) PHLPP1/2 overexpression itself was sufficient to trigger β-cell death and dysfunction; (3) mechanistically, the apoptosis-inducing effects of PHLPP1/2 were mediated not only through the inactivation of AKT pro-survival signaling but also through the activation of pro-apoptotic MST1 kinase, two downstream PHLPP substrates (both of these downstream signals have been implicated in β-cell failure and diabetes before); (4) chronic hyper-activation of mTORC1 was identified as a primary mechanism of PHLPPs upregulation, linking metabolic stress to ultimate β-cell death; and (5) genetic PHLPP1 inhibition protected against glucose intolerance and defective insulin secretion and promoted β-cell survival, β-cell proliferation, and compensatory β-cell mass expansion in two mouse models of diabetes and restored β-cell function and survival in human T2D islets.

Protein phosphorylation/de-phosphorylation is a key biochemical component of intracellular signaling pathways and has a crucial role in the transduction of signals to ultimately decide the fate of cells (Duncan et al., 2010; Takeda et al., 2011). PHLPP phosphatases potently promote cell death by inhibiting proliferative pathways (O'Neill et al., 2013). AKT kinase, which was the first well-established, physiological substrate of PHLPPs, is the master pro-survival kinase in β-cells. Activation of the AKT signaling pathways downstream of mitogen receptors, such as insulin, insulin growth factors (IGF family), and phosphoinositide 3-kinase (PI3K) (Boucher et al., 2014; Taniguchi et al., 2006), has a pivotal role in controlling β-cell growth, proliferation, and apoptosis. AKT-mediated phosphorylation of multiple substrates positively regulates insulin transcription, insulin secretion, and β-cell growth and survival (Assmann et al., 2009; Bernal-Mizrachi et al., 2001; Tuttle et al., 2001). PHLPPs directly de-phosphorylate AKT and inhibit its intrinsic catalytic activity. Consequently, PHLPP-induced inactivation of AKT results in apoptosis and inhibition of cell proliferation (Brognard et al., 2007; Gao et al., 2005). Defective phosphorylation of AKT at Ser473 is an important biochemical hallmark of human and rodent diabetic β-cells (Kim et al., 2012; Shirakawa et al., 2017; Wang et al., 2010; Yuan et al., 2017), but mechanistically, a key upstream element responsible for defective AKT signaling in the β-cell has not yet been well described.

In addition, the second PHLPP downstream target in the β-cell found in this study, MST1 (Jung et al., 2014; Qiao et al., 2010), is crucial for β-cell survival because it 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 β-cell survival and function in diabetes (Ardestani et al., 2014, 2019). In-depth investigations of the PHLPP downstream pathway demonstrate that PHLPP-AKT-MST1 constitutes a stress-sensitive survival pathway. Under acute stress conditions, AKT promoted cell survival by inhibiting MST1, but prolonged, unresolved metabolic stress upregulated PHLPPs with two obvious functional outputs: (1) a decrease in AKT

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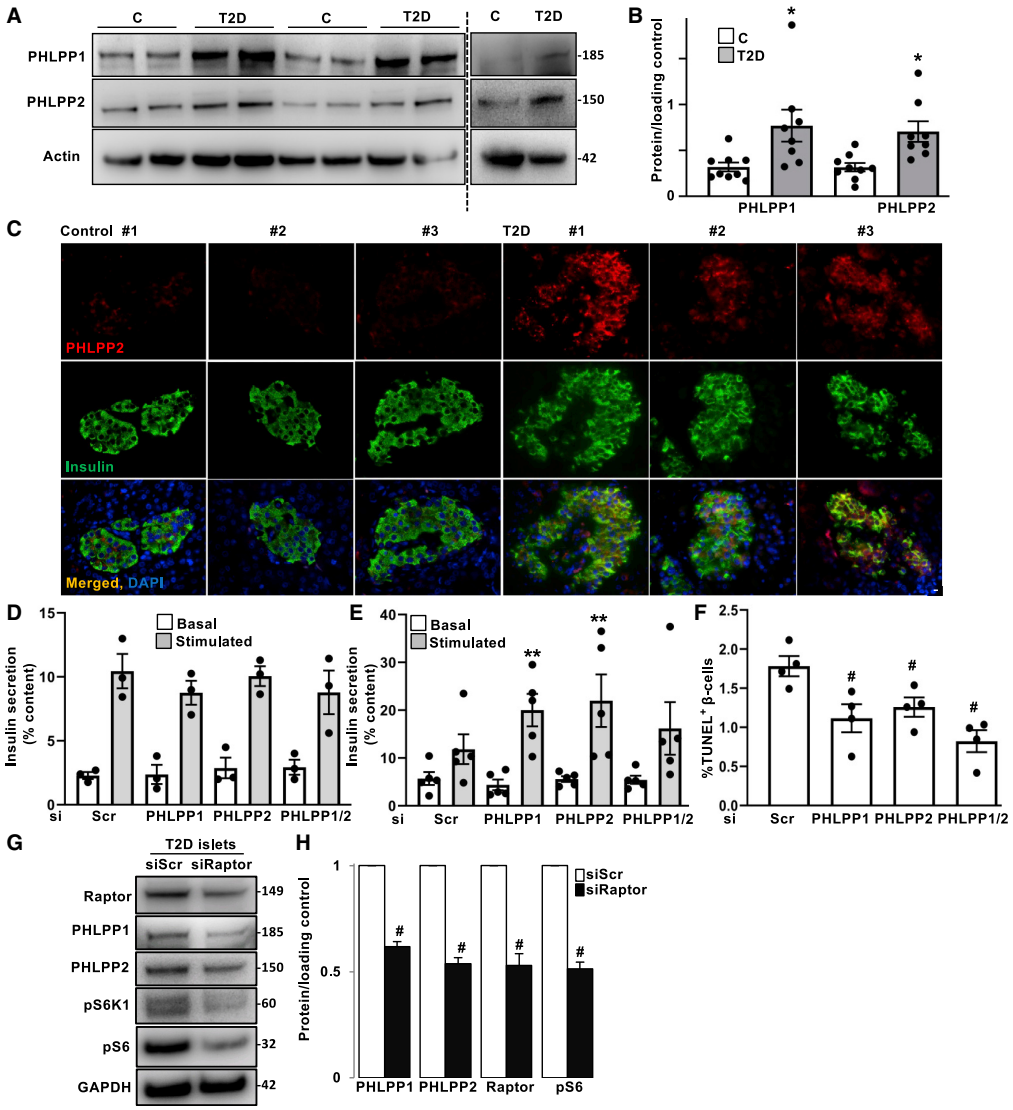

Figure 6. Genetic inhibition of PHLPP1/2 improved insulin secretion and β-cell survival in human islets from patients with T2D

(A and B) Representative western blots (A) and quantitative densitometry analysis (B) of human isolated islets from non-diabetic controls (n = 9) and patients with T2D (n = 8).

(C) Representative images of double immunostaining for PHLPP2 in red and insulin in green of pancreatic autopsy sections from non-diabetic controls (n = 4) and patients with T2D (n = 4; scale bar depicts 10 μm).

(D–F) Isolated human islets from non-diabetic individuals (D) and patients with T2D (E and F) were transfected with PHLPP1 and/or PHLPP2 siRNA or control siScr for 2 days. (D and E) Insulin secretion during 1 h of incubation with 2.8 mM (basal) and 16.7 mM (stimulated) glucose, normalized to insulin content (n = 3 controls; n = 5 T2D; each from three independent replicates, respectively). (F) Pooled TUNEL analysis (n = 4; each from three independent replicates, an average of 2,515 β cells were counted from each treatment condition).

(G and H) Representative western blots (G) and quantitative densitometry analysis (H) of human isolated islets from patients with T2D transfected with raptor siRNA or control siScr for 2 days (n = 3).

Data are expressed as means ± SEM. *p < 0.05 T2D versus control islets. **p < 0.05 siPHLPP1/2-transfected stimulated versus siPHLPP1/2-transfected basal. #p < 0.05 siPHLPP1/2- or siRaptor-transfected compared with siScr-transfected T2D islets.

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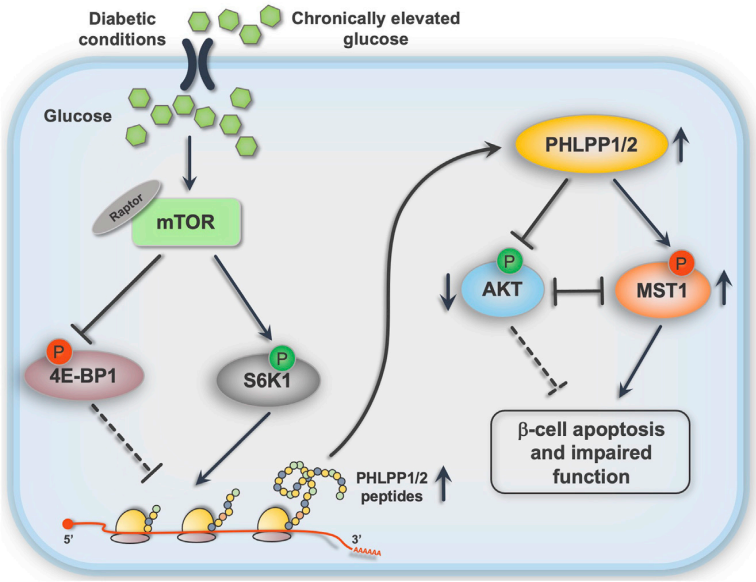

Figure 7. PHLPPing mTORC1 toward β-cell failure: Graphical summary of the results

Chronic metabolic stress leads to hyper-activation of mTORC1, promoting the PHLPP translational machinery, which leads to the triangle loop of PHLPP activity, AKT inhibition, and MST1 activation and, ultimately, to β-cell death and dysfunction.

activity through direct AKT-Ser473 de-phosphorylation, and (2) an increase in MST1 activity through direct MST1-Thr387 de-phosphorylation, which leads, in turn, to auto-phosphorylation of MST1-Thr183 and subsequent MST1 activation to induce apoptosis. Both these mechanisms cumulatively amplified pro-apoptotic MST1 signaling. This Thr387 site is similar to the site that is phosphorylated by AKT to inactivate MST1 and terminate apoptosis (Jang et al., 2007). Thus, PHLPP, AKT, and MST1 form an auto-inhibitory triangle that regulates β-cell apoptosis in a tightly controlled manner. Our data are fully in line with a recently published report that shows upregulation of PHLPP1/2 in INS-1 cells in response to elevated glucose and their link to AKT (Hribal et al., 2020).

mTORC1 signaling is an instrumental pathway in nutrient sensing and the integration of metabolic, energetic, and hormonal stimuli to control cellular metabolism, survival, and anabolic growth (Gonzalez and Hall, 2017; Mossmann et al., 2018; Saxton and Sabatini, 2017). Although physiological mTORC1 activation is necessary for the maintenance of β-cell growth, homeostasis, metabolic compensation, and insulin secretion, its long-term, sustained, aberrant activation—as illustrated in β-cells from patients with T2D and later in rodent islets (Jaafar et al., 2019; Yuan et al., 2017)—can promote β-cell failure, underscoring the dual and complex action of mTORC1 signals in pancreatic β-cells (Ardestani et al., 2018). A prerequisite for rescuing the β-cell from chronic metabolic stress would be to unravel the molecular mechanisms/targets underlying the "pathogenic arm" of inappropriate hyper-activated mTORC1 seen in diabetic β-cells. That would require opening the narrow mTORC1-based therapeutic window and avoiding compromising important mTORC1 homeostatic signals for β-cell homeostasis. We have identified PHLPP1/2 as mediators of the mTORC1-directed β-cell switch under diabetic conditions. Although sustained hyper-activation of mTORC1 upregulated PHLPPs and promoted β-cell apoptosis in metabolically stressed β-cells, both genetic and pharmacological interception of mTORC1 blocked PHLPP1/2 upregulation in response to nutritional stress. These data suggest that PHLPP is an important element of pathogenic mTORC1 signaling and that mTORC1 stimulation is essential for PHLPP1/2 to act as detrimental signals in stressed β-cells. The mTORC1-PHLPP1/2 axis offers a mechanistic link between glucotoxicity and dysregulation of β-cell survival and function. Only indirectly addressed here, the inhibition of PHLPP as an mTORC1 target could restore active AKT levels by halting the mTORC1-PHLPP-AKT loop.

Although suppression of PHLPP would be a desirable approach for β-cell mass preservation or expansion, the effects of prolonged PHLPPs inhibition should not be underestimated because of their function as tumor suppressors, as a logical consequence of apoptosis inhibition. PHLPP1-KO mice develop normally with no anatomical defects, consistent with previously reported studies showing that PHLPP1-KO mice are viable and show no overt changes in growth, anatomy, or development (Chen et al., 2013; Masubuchi et al., 2010). In addition, mice with systemic deletion/inhibition of PHLPP1 show promising neuro-, cardio-, and intestine-protection as well as tissue regeneration in several pathological settings (Chen et al., 2013; Hwang et al., 2018; Jackson et al., 2018; Moc et al., 2015; Wen et al., 2015; Zhang et al., 2019, 2020). In our long-term mouse studies, no tumorigenic features were observed in PHLPP1-KO mice; they live to a relatively old age without development of tumors. This may be due to compensatory actions by the other PHLPP isoform PHLPP2 or by the other AKT phosphatase PTEN (Chen et al., 2011; Molina et al., 2012). Moreover, PHLPP1 heterozygous mice are fully viable and show no growth abnormality compared with that of WT mice (Chen et al., 2011), and relative PHLPP1 deficiency can activate AKT signaling as efficiently as full PHLPP1 deletion (Moc et al., 2015). Nevertheless, although normal physiological functions and life span in PHLPP1-KO animals are generally not affected, PHLPP1 deletion accelerates tumor development in a mouse model of cancer (Li et al., 2014). Obviously, PHLPPs act as tumor suppressors and, thus, control oncogenic pathways. Although their permanent inactivation could lead to cancer development, it should be equally apparent that PHLPPs, and, in general, many other tumor suppressors, such as PTEN (Wang et al., 2010; Yang et al., 2014; Zeng

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et al., 2013), MST1 (Ardestani et al., 2014), P53 (Kung and Murphy, 2016), or $p27^{Kip1}$ (Uchida et al., 2005), are required for normal growth, compensatory proliferation, and regeneration of pancreatic β-cells, indicating that "regenerative pathways and oncogenic pathways are the same, differing only in their level, mechanism, and the duration of activation and safe regulatory mechanisms to turn on and off regenerative, and oncogenic pathways will need to be developed before regenerative approaches become accepted" (Wang et al., 2015). Pharmacological inhibition of PHLPPs—if proven to be selective with no or only little acceptable side effects—could recapitulate a moderate, but not absolute, PHLPP inhibition, which is unlikely to lead to uncontrolled cell proliferation and tumor development.

Another feature of PHLPP deletion observed in the study was the normalization of STZ-induced α-cell hyperplasia, which is a classical feature of diabetes (Cho et al., 2011; Dunning and Gerich, 2007; Moin and Butler, 2019; Yoon et al., 2003). The focus of this study was purely on β-cell survival mechanisms during diabetes progression; we cannot exclude a possibility that a β-to-α transdifferentiation might occur in diabetes and could also be regulated by PHLPP.

There is a critical need to develop therapeutic interventions to restore and maintain insulin secretion and β-cell mass in patients with T2D. Our multi-model approach not only shows PHLPPs as key phosphatases regulating β-cell survival but also identifies PHLPP-related up- and down-stream signal transductions that are activated by a pro-diabetic condition. PHLPPs deficiency restored normoglycemia and β-cell function and survival *in vivo* and *in vitro*. The identification of PHLPPs as key player in β-cell failure may have potential therapeutic relevance for the preservation and/or restoration of functional β-cell mass and glucose homeostasis in patients with T2D.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary materials. All raw data are available upon reasonable request from the authors.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109490.

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AUTHOR CONTRIBUTIONS

Conceptualization, supervision, and manuscript writing: A.A. and K.M.; methodology: B.L., A.A., and K.M.; formal analysis and investigation: B.L., K.A., H.I., S.K., S.G., B.S., A.P., S.A., A.J., S.R., M.K.M., M.K., H.L., T.Y., W.H., K.D.D.G., Z.A., and A.A.; resources, Q.Q., K.Y., and J.O.; funding acquisition: A.A. and K.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit anti-Ki-67	Dako; now Agilent, Santa Clara, CA, USA	#M7249; RRID:AB_2250503
rabbit anti-glucagon	Dako	#A0565; RRID:AB_10013726
guinea pig anti-insulin	Dako	#A0546; RRID:AB_2617169
mouse anti-NKX6.1	DSHB, University of Iowa, USA	#F55A12; RRID:AB_532379
rabbit anti-PHLPP2	Bethyl, TX, USA	#A300-661A; RRID:AB_2299551
rabbit anti-PDX1	Abcam, UK	#47267; RRID:AB_777179
rabbit anti-GLUT2	Chemicon, CA, USA),	#07-1402; RRID:AB_1587076
rabbit anti-HA-tag	Cell signaling technology (CST), Danvers, MA, USA	#2367; RRID:AB_10691311
rabbit anti-pAKT	CST	#9272; RRID:AB_329827
rabbit anti-cleaved caspase-3	CST	#9664; RRID:AB_2070042
rabbit anti-cleaved PARP	CST	rat specific; #9545; RRID:AB_2283565
rabbit anti-tubulin	CST	#2146; RRID:AB_2210545
rabbit anti-GAPDH	CST	#2118; RRID:AB_561053
rabbit anti–β-actin	CST	#4967; RRID:AB_330288
rabbit anti-GFP	CST	#2956; RRID:AB_1196615
rabbit anti-p4EBP1	CST	#2855; RRID:AB_560835
rabbit anti-pS6	CST	#4858; RRID:AB_916156
rabbit anti-pS6K	CST	#9234; RRID:AB_2269803
rabbit anti-Raptor	CST	#2280; RRID:AB_561245
rabbit anti-MST1	CST	#3682; RRID:AB_2144632
rabbit anti-AKT	CST	#9272; RRID:AB_329827
rabbit anti-pAKT	CST	#4058; RRID:AB_331168
rabbit anti-GST	CST	#2625; RRID:AB_490796
rabbit anti-pGSK3	CST	#9336; RRID:AB_331405
rabbit anti-PHLPP1	Proteintech, IL, USA	RRID: AB_2750897 #22789-I-AP
rabbit anti-pMST1(T183)	Abcam, UK	#ab79199; RRID:AB_2271183
horseradish-peroxidase-linked anti-rabbit	Jackson Immuno Research, PA, USA	#111-035-003; RRID:AB_2313567
horseradish-peroxidase-linked anti-mouse	Jackson	#115-035-003; RRID:AB_10015289
Cy3-conjugated donkey anti-mouse	Jackson	#715-165-150; RRID:AB_2340813
Cy3-conjugated anti-rabbit	Jackson	#711-165-152; RRID:AB_2307443
FITC-conjugated donkey anti-guinea pig	Jackson	#706-096-148; RRID:AB_2340454
Bacterial and virus strains		
adenoviruses Ad-LacZ	Vector Biolabs, PA, USA	#1080
Ad-h-PHLPP1	Vector Biolabs	N/A
Ad-h-PHLPP2	Vector Biolabs	#ADV-214159
Biological samples		
Human FFPE pancreatic sections from autopsy	this paper	N/A
Mouse FFPE pancreatic sections from autopsy	this paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Immobilon Western HRP Substrat	Millipore, MA, USA	#WBKLS0500
Protease and Phosphatase Inhibitors	Thermo Fisher Scientific, USA	#78440
RevertAid reverse transcriptase		#EP0451
S6K1 selective inhibitor PF-4708671	Calbiochem, USA	#S2163
Rapamycin	Calbiochem	#53123-88-9
IGF1	Calbiochem	#407251
recombinant human IL-1β	R&D Systems, USA	#201-LB
recombinant human IFN-γ	PeProTech, USA	#300-02
MHY1485	Selleck Chemicals, USA	#S7811
3-Benzyl-5-((2-nitrophenoxy)methyl)-dihydrofuran-2(3H)-one (3BDO)	J&K Scientific, Belgium	#1914077
recombinant human insulin	Sigma-Aldrich, USA	#91077C
cycloheximide	Sigma-Aldrich	#C4859-1ML
streptozotocin	Sigma-Aldrich	#S0130
Phenol:Chloroform:Isoamyl Alcohol	Sigma-Aldrich	#77617
Liberase TM	Roche, Switzerland	#05401119001
jetPRIME® transfection reagent	Polyplus, France	#114-75
in vivo-jetPEI	Polyplus	#201-50G
Vectashield with 4′6-diamidino-2-phenylindole (DAPI)	Vector Labs, USA	#H-1200-10
TriFast	PEQLAB Biotechnologie, Germany	#30-2010
Lipofectamine 2000	Invitrogen, USA	#11668019
Critical commercial assays		
translatome analysis: AHARIBO RNA	IMMAGINA Biotechnology, Italy	#AHA003-R
Pierce BCA Protein Assay	Thermo Fisher Scientific, USA	#23225
Insulin ELISA Assay	ALPCO Diagnostics, USA	#80-INSMSU-E01
In situ Cell Death Detection Kit, TMR red	Roche, Switzerland	#12156792910
VECTASTAIN ABC Kit	Vector Labs, USA	#PK-4000
Deposited data		
N/A		
Experimental models: Cell lines		
rat β-cell line INS-1E	Laboratory of Claes Wollheim, University of Geneva	RRID: CVCL_0351
Mouse embryonic fibroblasts (MEFs) isolated from Tuberous sclerosis complex 2 knock-out (MEF-TSC2-KO) and respective WT mice	Laboratory of Gil Leibowitz, Hadassah University	N/A
Mouse embryonic fibroblasts (MEFs) isolated from PHLPP1 knock-out (MEF-PHLPP1-KO) and respective WT mice	Laboratory of Alexandra Newton, UCSD	N/A
Experimental models: Organisms/strains		
PHLPP1-KO mice	Laboratory of Alexandra Newton, UCSD	RRID: MGI:5795609
leptin receptor deficient mice $Lepr^{db/+}$(db/$^{+}$), $Lepr^{db/db}$ (db/db)	Jackson Laboratory, ME, USA	#000642; BKS.Cg-Dock7^{m} +/+ $Lepr^{db}$/J
Isolated mouse islets	this paper	N/A
Isolated human islets	this paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
rat PHLPP1 sequences 5'CAGCUUGACCUGC GAGACA3'; 5'GUGAAUAACUUCCGUGACA3'; 5' UAAUAGUAGUCUCCGGAAA3'; 5'GAAUGUAC AAUGUCCGAAA3'	ON-TARGETplus siRNAs, Dharmacon, CO, USA	#L-094929-02
rat PHLPP2 sequences 5'ACAAAUGGGCUGA GCGCUU3'; 5'UAGUCUGAGUCUUCGGAAA3'; 5' GCAUCUAUAACGUCCGCAA3'; 5'CCGUGGAC CUCUCGUGUUA3'	ON-TARGETplus	#L-104590-02
human PHLPP1 sequences 5'GAAUGUAUAAU GUCCGUAA3'; 5'GAUCUAAGGUUGAACGUAA3'; 5' GGAAUCAACUGGUCACAUU3'; 5'GAUAUUGG CCAUAAUCAAA3',	ON-TARGETplus	#L-019103-00
human PHLPP2 sequences 5'CCUAUAUUGU UAUGCGAGA3'; 5'CCGUGGAUCUCUCGUGUUA3'; 5' GAUCCAGUUUGUAGACCUA3'; 5'UGCAACGA CUUGACAGAAA3'	ON-TARGETplus	#L-022586-01
human Raptor sequences 5'UGGCUAGUCUGU UUCGAAA3'; 5'CACGGAAGAUGUUCGACAA3'; 5' AGAAGGGCAUUACGAGAUU3'; 5'UGGAGAAGC GUGUCAGAUA3'	ON-TARGETplus	#L-004107-00
rat Raptor sequences 5'GAGCUUGACUCCAG UUCGA3', 5'GCUAGGAACCUGAACAAAU3', 5'GCA CACAGCAUGGGUGGUA3', 5'GAAUCAUGAGG UGGUAUAA3'	ON-TARGETplus	#L-086862-02
rat MST1 sequences 5'CUCCGAAACAAGACG UUAA3'; 5'CGGCAGAAAUACCGCUCCA; 5'CGAG AUAUCAAGGCGGGAA3'; 5'GGAUGGAGACUA CGAGUUU3'	ON-TARGETplus	#L-093629-02
rat S6K1 sequences 5'GGCCAGAGCACCUGC GUAU3'; 5'ACAAAAGCAGAGCGGAAUA3'; 5'GCGC CUGACUUCCGACACA3'; 5'CGGAGAACAUCA UGCUUAA3'	ON-TARGETplus	#L-099323-02
human PHLPP1	TaqMan® Gene Expression Assays, Applied Biosystems, CA, USA	#Hs01597875_m1
human PHLPP2	Applied Biosystems	#Hs00982295_m1
mouse Phlpp1	Applied Biosystems	#Mm01295850_m1
mouse Phlpp2	Applied Biosystems	#Mm01244267_m1
rat Phlpp1	Applied Biosystems	#Rn00572211_m1
rat Phlpp2	Applied Biosystems	#Rn01431647_m1
human TUBA1A	Applied Biosystems	#Hs00362387_m1
mouse Ppia	Applied Biosystems	#Mm03024003_g1
mouse Tuba1a	Applied Biosystems	#Mm00846967_g1
rat Ppia	Applied Biosystems	#Rn00690933_m1
human PPIA	Applied Biosystems	#Hs99999904_m1
rat Tuba1a	Applied Biosystems	#Rn01532518_g1
Recombinant DNA		
Phospho-mimetic AKT1 mutant; pCDNA3-HA-AKT1 S473D	Laboratory of Wenyi Wei, Harvard Medical School Liu et al., 2014	N/A
Kinase-dead MST1; pCMV-MST1-K59R	Laboratory of J. Sadoshima and Y. Maejima; Yamamoto et al., 2003	N/A

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Phospho-mimetic MST1 mutant; GST-MST1 T387E	Laboratory of Qi Qi and Keqiang Ye; Jang et al., 2007	N/A
active form of AKT1; Myr-HA AKT1	Ramaswamy et al., 1999	William Sellers, Broad Institute of MIT http://n2t.net/addgene:9008; RRID: Addgene_9008
pcDNA3 HA-PHLPP1 full length	Warfel et al., 2011	Alexandra Newton, UCSD http://n2t.net/addgene:37100; RRID: Addgene_37100
pcDNA3-HA-PHLPP2	Brognard et al., 2007	Alexandra Newton, UCSD http://n2t.net/addgene:22403; RRID: Addgene_22403
Constitutively active form of S6K1; pRK7-HA-S6K1-F5A-E389-R3A	Schalm and Blenis, 2002	John Blenis, Weill Cornell Medicine http://n2t.net/addgene:8991; RRID: Addgene_8991
Software and algorithms		
Vision Works LS Image Acquisition and Analysis software Version 6.8	UVP BioImaging Systems, CA, USA	https://www.labortechnik.com/en/vision-works-ls/analysis-software
NIS-Elements software, v3.22.11	Nikon GmbH, Germany	https://www.nikon.com/products/microscope-solutions/support/download/software/imgsfw/nis-f_v4600064.htm
GraphPad Prism v8.4.3	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
Biocoat Collagen I coated dishes	Corning, ME, USA	#356400
CMRL-1066	Invitrogen, USA	#11530037
RPMI-1640	Sigma-Aldrich, MO, USA	#R8758
DMEM high glucose		#D6429
The Applied Biosystems StepOne Real-Time PCR system	Applied Biosystems, USA	N/A
Nikon MEA53200	Nikon GmbH, Germany	N/A
Glucometer FreeStyle Lite	Abbott, USA	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kathrin Maedler (kmaedler@uni-bremen.de)

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data generated from this study are included in this paper. All raw data reported in this paper will be shared by the lead contact upon reasonable request. This paper does not report original code.Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Cell culture, treatment and islet isolation

Human islets were isolated from pancreases of nondiabetic organ donors or donors with type 2 diabetes (both from males and females) at Universities of Illinois at Chicago, Wisconsin, Lille or ProdoLabs and cultured on extra cellular matrix (ECM)-coated dishes (Novamed, Israel) or on Biocoat Collagen I coated dishes (#356400, Corning, ME, USA). The clonal rat β-cell line INS-1E was kindly

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provided by Claes Wollheim (Geneva & Lund University). Mouse embryonic fibroblasts (MEFs) isolated from Tuberous sclerosis complex 2 knock-out (MEF-TSC2-KO) and respective WT mice (generously provided to our lab by Gil Leibowitz, Hadassah-Hebrew University Medical Center, Jerusalem) or from PHLPP1 knock-out (MEF-PHLPP1-KO) and respective WT mice (generously provided to our lab by Alexandra Newton, UCSD) were cultured in complete DMEM (Sigma-Aldrich, MO, USA) medium at 25 mM glucose. Human islets were cultured in complete CMRL-1066 (Invitrogen, CA, USA) medium at 5.5 mM glucose and mouse islets and INS-1E cells in complete RPMI-1640 (Sigma-Aldrich, MO, USA) medium at 11.1 mM glucose as described previously (Ardestani et al., 2014). Mouse islets were isolated by pancreas perfusion with a Liberase TM (#05401119001; Roche, Switzerland) solution (Ardestani et al., 2019) according to the manufacturer's instructions and digested at 37°C, followed by washing and handpicking. Human and mouse islets and INS-1E cells were exposed to complex diabetogenic conditions: 22.2 mM glucose, in combination with 0.5 mM palmitic acid, or the mixture of 2 ng/mL recombinant human IL-1β (R&D Systems, MN, USA) plus 1,000 U/ml recombinant human IFN-γ (PeProTech, NJ, USA) for 1-3 days. Palmitic acid was dissolved as described previously (Maedler et al., 2001). In some experiments, cells or primary islets were additionally cultured with 100 nM Rapamycin or 10 μM S6K1 selective inhibitor PF-4708671 (Calbiochem, CA, USA) for 1-2 days, 10 to 20 μM 3-Benzyl-5-((2-nitrophenoxy)methyl)-dihydrofuran-2(3H)-one (3BDO) (J&K Scientific, Belgium) for 3h, 25 μM MHY1485 (Selleck Chemicals, TX, USA) for 3h, 100 ng/ml IGF1 (#407251; Calbiochem, CA, USA), 100 nM recombinant human insulin, 50 μg/ml cycloheximide (CHX) or 1 mM streptozotocin (STZ) (all Sigma-Aldrich). All human islet experiments were performed in the islet biology laboratory, University of Bremen. Human islets were distributed by the two JDRF and NIH supported approved coordination programs in Europe (Islet for Basic Research program; European Consortium for Islet Transplantation ECIT) and in the US (Integrated Islet Distribution Program IIDP) (Hart and Powers, 2019). Autopsy pancreases from non-diabetic controls and from patients with T2D were obtained from the National Disease Research Interchange (NDRI).

Ethical approval for the use of human islets had been granted by the Ethics Committee of the University of Bremen. The study complied with all relevant ethical regulations for work with human cells for research purposes. Organ donors are not identifiable and anonymous, such approved experiments using human islet cells for research is covered by the NIH Exemption 4 (Regulation PHS 398).

Animals

For multiple low dose streptozotocin (MLD-STZ) experiments, 8- to 10-week old male PHLPP1-KO mice (RRID: MGI:5795609; generously provided by Alexandra Newton, UCSD) (Masubuchi et al., 2010) on a C57BL/6J genetic background and their WT littermates were injected with multiple low-dose STZ (40 mg/kg body weight), freshly dissolved in 50 mM sodium citrate buffer, for five consecutive days. For the high-fat, high-sucrose diet (HFD) experiments, 8-week-old male WT and PHLPP1-KO mice 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 HFD (Surwit Research Diets, New Brunswick, NJ, containing 58, 26 and 16% calories from fat, carbohydrate and protein, respectively) for 17 weeks. For both groups, random blood was obtained from the tail vein of nonfasted mice, and glucose was measured using a Glucometer (FreeStyle; Abbott, IL, USA). Heterozygous leptin receptor deficient mice on the C57BLKS/J background ($Lepr^{db/+}$, $db/^{+}$) were purchased from Jackson Laboratory, ME, USA. By breeding of these mice, we obtained diabetic $Lepr^{db/db}$ (db/db) as well as non-diabetic heterozygous $Lepr^{db/+}$ (db/+) mice.

All mice used in the experiments 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 NIH animal care guidelines, §8 German animal protection law, German animal welfare legislation and with the guidelines of the Society of Laboratory Animals (GV-SOLAS) and the Federation of Laboratory Animal Science Associations (FELASA).

Ethical approval for the mouse experiments had been granted by the Bremen Senate (Senator for Science, Health and consumer protection) and we have complied with all relevant ethical regulations for animal testing and research.

METHOD DETAILS

Glucose and insulin tolerance tests and insulin secretion

For intraperitoneal glucose tolerant tests (ipGTT), mice were fasted overnight for 12h and injected i.p. with glucose (B.Braun, Germany) at a dose of 1g/kg body weight. Blood samples were collected at time points 0, 15, 30, 60, 90, and 120 min for glucose measurements by using a Glucometer (FreeStyle; Abbott, IL, USA). For i.p. insulin tolerance tests (ipITT), mice were initially fasted for a period of 4 h followed by recombinant human insulin injection (Novo Nordisk, Denmark) at a dose of 0.75 U/kg body weight. Glucose concentration was determined with the Glucometer at time points 0, 15, 30, 60 and 90 min. Blood samples for insulin secretion was collected before (0 min) and after (15 and 30 min) i.p. injection of glucose (2g/kg body weight) and measured by using ultrasensitive mouse ELISA kit (ALPCO Diagnostics, NH, USA).

Plasmids and siRNAs

To knock down PHLPP1, PHLPP2, MST1, raptor and S6K1 SMARTpool technology from Dharmacon, CO, USA was used. A mix of ON-TARGETplus siRNAs directed against the following sequences: rat PHLPP1 (#L-094929-02) sequences CAGCUUGACCUGCGA GACA; GUGAAUAACUUCCGUGACA; UAAUAGUAGUCUCCGGAAA; GAAUGUACAAUGUCCGAAA, rat PHLPP2 (#L-104590-02) sequences ACAAAUGGGCUGAGCGCUU; UAGUCUGAGUCUUCGGAAA; GCAUCUAUAACGUCCGCAA; CCGUGGACCUCUCGU GUUA, human PHLPP1 (#L-019103-00) sequences GAAUGUAUAAUGUCCGUAA; GAUCUAAGGUUGAACGUAA; GGAAUCAACUG

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GUCACAUU; GAUAUUGGCCAUAAUCAAA, human PHLPP2 (#L-022586-01) sequences CCUAUAUUGUUAUGCGAGA; CCGUGG AUCUCUCGUGUUA; GAUCCAGUUUGUAGACCUA; UGCAACGACUUGACAGAAA, human Raptor (#L-004107-00) sequences UGGCUAGUCUGUUUCGAAA; CACGGAAGAUGUUCGACAA; AGAAGGGCAUUACGAGAUU; UGGAGAAGCGUGUCAGAUA, rat Raptor (#L-086862-02) sequences GAGCUUGACUCCAGUUCGA, GCUAGGAACCUGAACAAAU, GCACACAGCAUGGGUGGUA, GAAUCAUGAGGUGGUAUAA, rat MST1 (#L-093629-02) sequences CUCCGAAACAAGACGUUAA; CGGCAGAAAUACCGCUCCA; CGAGAUAUCAAGGCGGGAA; GGAUGGAGACUACGAGUUU, and rat S6K1 (#L-099323-02) sequences GGCCAGAGCACCUGC GUAU; ACAAAAGCAGAGCGGAAUA; GCGCCUGACUUCCGACACA; CGGAGAACAUCAUGCUUAA. An ON-TARGETplus nontargeting siRNA pool (Scramble; siScr) served as controls.

Following plasmids have been used: constitutively active form of AKT1; Myr-HA AKT1 was a gift from William Sellers (Addgene plasmid # 9008; http://addgene.org/9008; RRID: Addgene_9008) (Ramaswamy et al., 1999). Phospho-mimetic AKT1 mutant; pCDNA3-HA-AKT1 S473D was a gift from Wenyi Wei (Harvard Medical School, USA) (Liu et al., 2014). Kinase-dead MST1; pCMV-MST1-K59R was a gift from J. Sadoshima and Y. Maejima (Rutgers New Jersey Medical School, USA) (Yamamoto et al., 2003). Phospho-mimetic MST1 mutant; GST-MST1 T387E from Qi Qi and Keqiang Ye (Emory University School of Medicine, USA) (Jang et al., 2007). pcDNA3 HA-PHLPP1 full length was a gift from Alexandra Newton (Addgene plasmid # 37100; http://addgene.org/37100; RRID: Addgene_37100) (Warfel et al., 2011). pcDNA3-HA-PHLPP2 was a gift from Alexandra Newton (Addgene plasmid # 22403; http://addgene.org/22403; RRID: Addgene_22403) (Brognard et al., 2007). Constitutively active form of S6K1: pRK7-HA-S6K1-F5A-E389-R3A was a gift from John Blenis (Addgene plasmid # 8991; http://addgene.org/8991; RRID: Addgene_8991) (Schalm and Blenis, 2002). GFP plasmid was used as a control.

Transfection

To achieve silencing and overexpression in human islets and INS-1E cells previously described protocol was used (Ardestani et al., 2014). In brief, human islets were dispersed into smaller cell aggregates using accutase (PAA) to increase transfection efficiency and subsequently cultured on ECM- or Collagen I- coated dishes for 1-2 days. To deliver desired siRNA/DNA into dispersed isolated islets as well as INS-1E cells two different transfection methods were used. First, partially dispersed islets or INS-1E cells were pre-incubated in transfection Ca^{2+}-KRH medium (KCl 4.74 mM, KH_2PO_4 1.19 mM, $MgCl_26H_2O$ 1.19 mM, NaCl 119 mM, $CaCl_2$ 2.54 mM, $NaHCO_3$ 25 mM, HEPES 10 mM) for 1h. After that lipoplexes (#11668019; Lipofectamine 2000, Invitrogen, CA, USA)/siRNA ratio 1:20 pmol or lipoplexes/DNA ratio 2.5:1) were added to Ca^{2+}-KRH medium for 6h to transfect the islets or INS-1E cells. After transfection, medium was replaced for fresh CMRL-1066 or RPMI-1640 medium containing 20% FCS. Second, jetPRIME transfection reagent (#114-75; Polyplus transfection, France) was mixed with jetPRIME buffer and siRNA/DNA according to manufacturer's instructions. The jetPRIME-siRNA/DNA complexes were then added to complete CMRL-1066 or RPMI-1640 to transfect dispersed human islets or INS-1E cells. Efficient transfection was evaluated based on western blot, qPCR and fluorescent microscopy.

In vivo nucleic acid delivery

A commercially available cationic polymer transfection reagent *in vivo*-jetPEI (#201-50G; Polyplus transfection, France) was used to deliver HA-conjugated PHLPP1- and PHLPP2-expressing constructs via i.p. injection according to manufacturer's instructions. Briefly, 50 μg of each PHLPP1 and PHLPP2 or 100 μg control GFP plasmids were diluted in 200 μL of 5% glucose solution and mixed with *in vivo*-jetPEI transfection reagent based on the recommended ionic balance (N/p = 6-8). For an optimum of PHLPP1/2 overexpression, the plasmid/jetPEI mixture was i.p. injected into C57BL/6J male mice (Jackson Laboratory) five times every alternate day for 10 days. Mice were sacrificed 24h after last injection and pancreas/islets isolated.

Adenovirus transduction

The adenoviruses control Ad-LacZ as well as Ad-h-PHLPP1 and Ad-h-PHLPP2 expressing human PHLPP1 and PHLPP2 were purchased from Vector Biolabs, PA, USA. For transduction, isolated human or mouse islets or INS-1E cells were first plated for 1 day followed by infection at a multiplicity of infection (MOI) of 20 (for INS-1E) or 100 (for human and mouse islets) for 4h in CMRL-1066 or RPMI-1640 medium without FCS. After 4h incubation, adenovirus was washed off with 1xPBS and replaced by fresh complete medium. Human islets or INS-1E cells were additionally incubated for 1-3 days.

Glucose-stimulated insulin secretion (GSIS)

Human and mouse islets were pre-incubated in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose for 30 min followed by fresh KRB containing 2.8 mM glucose for 1h (basal) and additional 1h in KRB containing 16.7 mM glucose (stimulated). Islets were washed with 1xPBS and lysed with RIPA buffer for measuring total insulin content. Insulin was determined using human and mouse insulin ELISA (ALPCO Diagnostics, NH USA). Secreted insulin was normalized to insulin content.

Immunohistochemistry

Mouse pancreases were dissected and fixed in 4% formaldehyde at 4°C for 8h and dehydrated before embedding in paraffin. Human pancreatic sections obtained from autopsy from both male and female organ donors and mouse sections (both 2 μm) were deparaffinized, rehydrated and incubated overnight at 4°C with rabbit anti-Ki-67 (#M7249; Dako), rabbit anti-HA-tag (#2367;CST), rabbit anti-pAKT (#9272;CST), mouse anti-NKX6.1 (#F55A12; DSHB, University of Iowa, USA [Ben-Othman et al., 2017]), rabbit anti-PHLPP2

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(#A300-661A, Bethyl, TX, USA), rabbit anti-PDX1 antibody (#47267; Abcam, UK), rabbit anti-GLUT2 antibody (#07-1402; Chemicon, CA, USA), rabbit anti-glucagon (#A0565; Dako) or for 2 h at room temperature with anti-insulin (#A0546; Dako), followed by fluorescein isothiocyanate (FITC)- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, PA, USA). Slides were mounted with Vectashield with 4′6-diamidino-2-phenylindole (DAPI) (Vector Labs, CA, USA). Pancreatic β-cell apoptosis was analyzed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique according to the manufacturer's instructions (*In situ* Cell Death Detection Kit, TMR red; Roche, Switzerland) and double stained for insulin. Fluorescence was analyzed using a Nikon MEA53200 (Nikon GmbH, Germany) microscope, and images were acquired using NIS-Elements software from 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 (VECTASTAIN ABC Kit; Vector Labs, USA) were determined by computer-assisted measurements by using a Nikon MEA53200 (Nikon GmbH, Germany) microscope, and images were acquired by using NIS-Elements software from Nikon. Mean percent β-cell fraction per pancreas was calculated as the ratio of insulin-positive and whole pancreatic tissue area. Pancreatic β-cell mass was obtained by multiplying the β-cell fraction by the weight of the pancreas (Ardestani et al., 2014).

Western blot analysis

Human or mouse islets and INS-1E cells were washed twice with ice-cold PBS and lysed with RIPA lysis buffer containing Protease and Phosphatase Inhibitors (Thermo Fisher Scientific (TFS), MA, USA). Protein concentrations were measured by the BCA protein assay (TFS). Proteins were separated by size on NuPAGE 4%–12% Bis-Tris gel (Invitrogen; CA, USA) and electrically transferred into PVDF membranes. Membranes were blocked at room temperature using mixture of 2.5% milk (Cell Signaling Technology (CST), MA, USA) and 2.5% BSA for 1h and incubated overnight at 4°C with rabbit anti-cleaved caspase-3 (#9664), rabbit anti-cleaved PARP (rat specific; #9545), rabbit anti-tubulin (#2146), rabbit anti-GAPDH (#2118), rabbit anti-β-actin (#4967), rabbit anti-GFP (#2956), rabbit anti-HA (#2367), rabbit anti-p4EBP1 (#2855), rabbit anti-pS6 (#4858), rabbit anti-pS6K (#9234), rabbit anti-Raptor (#2280), rabbit anti-MST1 (#3682), rabbit anti-AKT (#9272), rabbit anti-pAKT (#4058), rabbit anti-GST (#2625), rabbit anti-pGSK3 (#9336) (all CST), rabbit anti-PHLPP1 (RRID: AB_2750897 #22789-I-AP, Proteintech, IL, USA), rabbit anti-PHLPP2 (#A300-661A; Bethyl, TX, USA), rabbit anti-pMST1(T183) (#ab79199, Abcam, UK) and rabbit anti-GLUT2 antibody (#07-1402; Chemicon, CA, USA). All primary antibodies were used at 1:1000 dilution in 1xTris-buffered saline plus Tween-20 (1xTBS-T) containing 5% BSA. Additionally, membranes were incubated with horseradish-peroxidase-linked anti-rabbit (Jackson ImmunoResearch, PA, USA) and developed using Immobilon Western HRP chemiluminescence assay system (#WBKLS0500; Millipore, MA, USA). Analysis of the immunoblots was performed using Vision Works LS Image Acquisition and Analysis software Version 6.8 (UVP BioImaging Systems, CA, USA).

Protein degradation analysis

INS-1E cells left untreated or treated with high glucose at 22 mM. At 2 days after incubation, cells were treated with 50 μg/ml translation initiation inhibitor cycloheximide (CHX) to the medium at the times indicated and the lysates were subjected to western blotting.

qPCR analysis

Total RNA was isolated from cultured human or mouse islets or INS-1E cells using TriFast (PEQLAB Biotechnologie, Germany). cDNA synthesis (RevertAid reverse transcriptase, Thermo Fisher Scientific (TFS), MA, USA) and quantitative RT-PCR was performed as previously described (Ardestani et al., 2014). The Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, CA, USA) with TaqMan® Fast Universal PCR Master Mix for TaqMan assays (Applied Biosystems) were used for analysis. TaqMan® Gene Expression Assays were used for *PHLPP1* (#Hs01597875_m1), *PHLPP2* (#Hs00982295_m1), *PPIA* (#Hs99999904_m1), and *TUBA1A* (#Hs00362387_m1) for human, *Phlpp1* (#Mm01295850_m1), *Phlpp2* (#Mm01244267_m1), *Ppia* (#Mm03024003_g1), and *Tuba1a* (#Mm00846967_g1) for mouse, and *Phlpp1* (#Rn00572211_m1), *Phlpp2* (#Rn01431647_m1), *Ppia* (#Rn00690933_m1) and *Tuba1a* (#Rn01532518_g1) for rat. qPCR was performed and analyzed by the Applied Biosystems StepOne Real-Time PCR system. The ΔΔCT method was used to analyze the relative changes in gene expression.

Translatome analysis

Active polyribosomes with associated mRNAs and nascent peptides for translatome analysis were isolated after an adapted protocol of AHARIBO RNA (#AHA003-R; IMMAGINA Biotechnology, Italy) (Figure 3F). INS-1E cells were cultured till 80% confluency in complete RPMI-1640 medium supplemented with 22.2 mM glucose for 1h. Pancreatic islets from mice fed a ND or HFD were isolated and cultured in complete RPMI-1640 medium. Cells were exposed to L-methionine-free medium (Invitrogen, CA, USA) for 40 min to deplete methionine reserves, followed by 1h treatment with 0.5 mM L-azidohomoalanine (AHA). After AHA incorporation, translation was blocked using sBlock for 10 min and cells were lysed in 1% sodium deoxycholate, 5 U/ml DNase 1, sBlock, 1x proteinase and phosphatase inhibitor cocktail, 200 U/ml RiboLock RNase Inhibitor. 5% of the lysate was saved for input. The remaining lysate was mixed with pre-functionalized magnetic beads (magnetic beads+ biotinylated alkyne ligand) for a chemo-selective "click reaction" between an azide and an alkyne for the effective pull-down of active ribosome complexes. After pull-down, ribosome complexes were digested using proteinase K (VWR, PA, USA) for 75 min. Ribosome associated mRNAs were then extracted with

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Phenol:Chloroform:Isoamyl Alcohol (Sigma-Aldrich) and used for qPCR analysis. The modified version of the ΔΔCT method was used to calculate a fold change of Up/downregulation of target gene at the translational level.

QUANTIFICATION AND STATISTICAL ANALYSIS

At least three independent biological replica (referred to "n") were used for human and mouse islets (from different donors/islet preparations from independent experiments), or INS-1E cells (independent experiments) or mice as reported in all figure legends, unless otherwise stated. Data are presented as means ± SEM. Mean differences were determined by Student's t tests. p value < 0.05 was considered statistically significant.

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Supplemental information

Inhibition of PHLPP1/2 phosphatases rescues pancreatic β-cells in diabetes

Blaz Lupse, Karthika Annamalai, Hazem Ibrahim, Supreet Kaur, Shirin Geravandi, Bhavishya Sarma, Anasua Pal, Sushil Awal, Arundhati Joshi, Sahar Rafizadeh, Murali Krishna Madduri, Mona Khazaei, Huan Liu, Ting Yuan, Wei He, Kanaka Durga Devi Gorrepati, Zahra Azizi, Qi Qi, Keqiang Ye, Jose Oberholzer, Kathrin Maedler, and Amin Ardestani

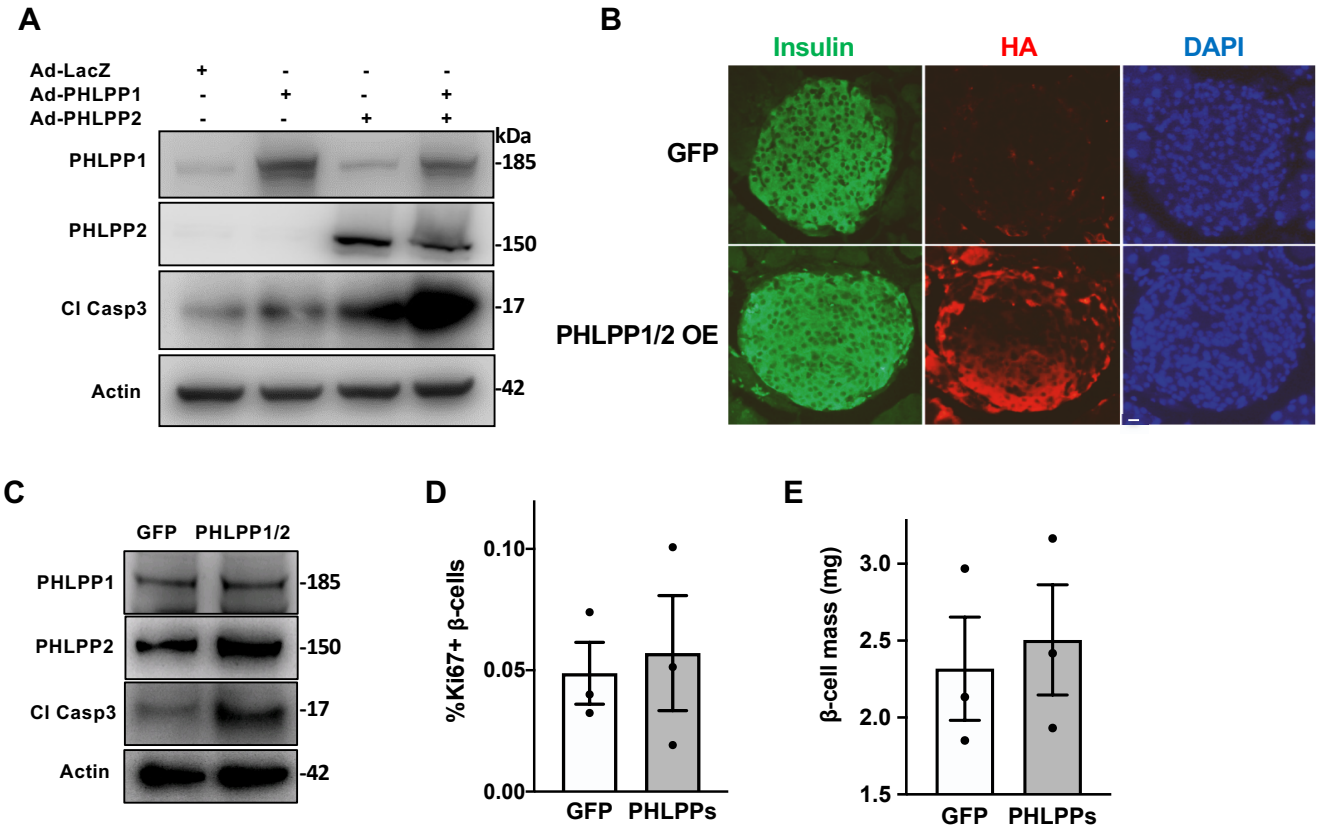

Figure S1. PHLPP1/2 overexpression induces β-cell apoptosis. Related to Figure 1. (A) Representative Western blots of INS-1E β-cells transduced with LacZ control or PHLPP1 and/or PHLPP2 adenoviruses for 48h (n=3). (B) Representative images of triple immunostaining for HA in red, insulin in green and DAPI in blue in pancreatic sections (n=3) (scale bar depicts 50µm), (C) representative Western blots of isolated islets (n=1 independent experiments; each pooled from 3 mice/ condition) and (D) analysis of Ki67+/insulin+ β-cells and (E) β-cell mass from *in vivo* plasmid/jetPEI GFP- or PHLPP1/2-transfected mice over 10 days (n=3). Data are expressed as means ± SEM

Figure S1

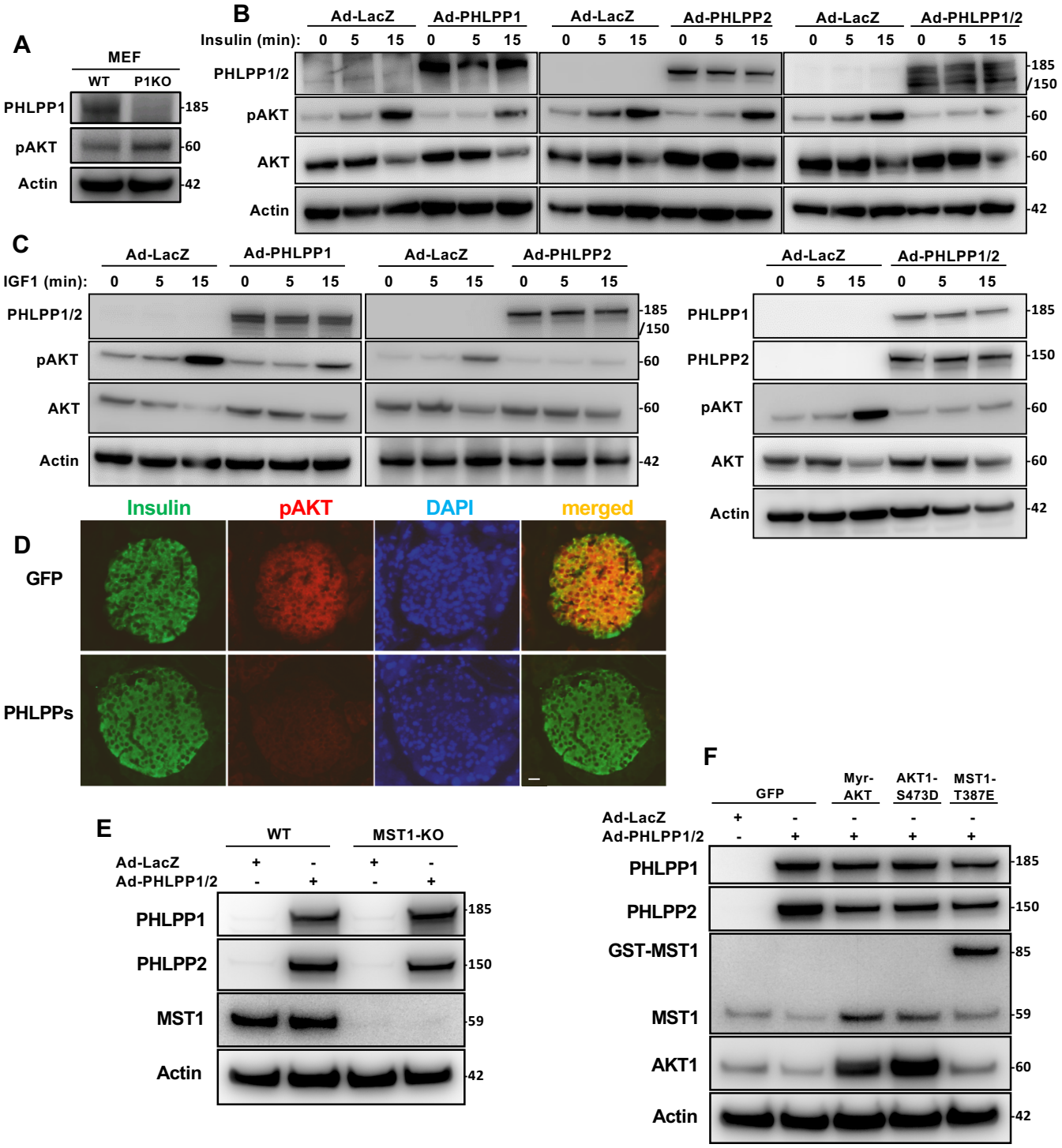

Figure S2. PHLPP-AKT-MST1 crosstalk. Related to Figure 2. (A) Western blots of WT and PHLPP1-KO MEF cells (n=5). (B,C) Representative Western blots of INS-1E β-cells transduced with LacZ control or PHLPP1 and/or PHLPP2 adenoviruses and then subjected to (B) insulin (100nM) or (C) IGF-I (100 µg/ml) stimulation (n=2-3). (D) Representative images of triple immunostaining for pAKT in red, insulin in green and DAPI in blue in pancreatic sections from GFP- or PHLPP1/2-transfected mice; scale bar depicts 50µm (n=3). (E) Representative Western blots of isolated islets from MST1-KO mice and their WT littermates after transduction with adenoviruses for LacZ (control) or PHLPP1/2 (n=1 independent experiments; each pooled from 4 mice/ condition). (F) Representative Western blots of isolated human islets overexpressed with adenoviruses for LacZ (control) or PHLPP1/2 and transfected with GFP (control) or Myr-AKT1 or HA-tagged AKT-S473D, or MST1-T387E plasmids (n=1).

Figure S2

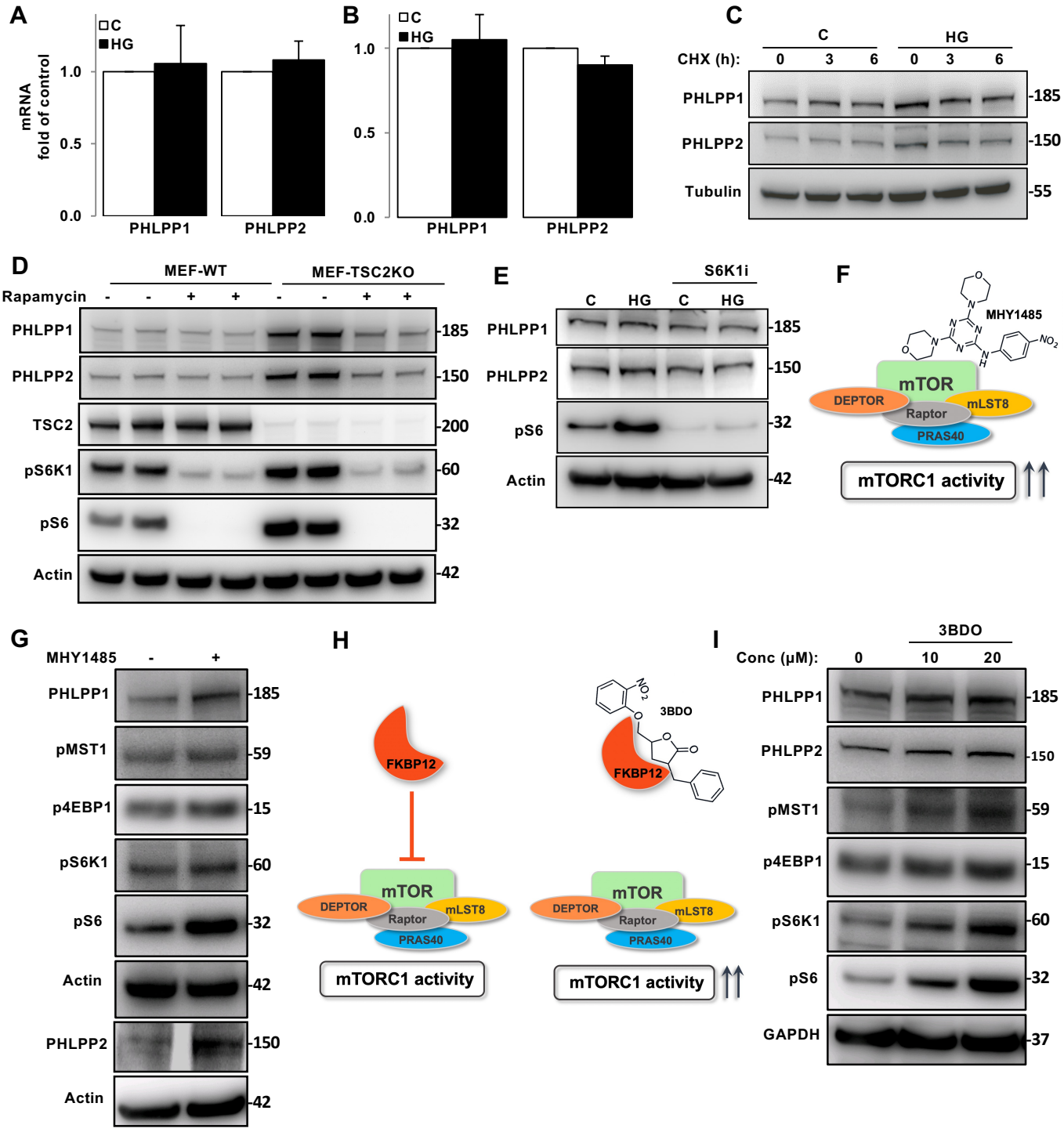

Figure S3. mTORC1 induces PHLPPs. Related to Figure 3. (A,B) qPCR for PHLPP1 or PHLPP2 mRNA expression in INS-1E β-cells (A; n=4) or isolated human islets (B; n=3) treated with high glucose (22mM) for 1 or 3 days respectively. Data are expressed as means ± SEM. (C) Representative Western blots of INS-1E β-cells treated with high glucose (22mM) for 2 days and then treated with 50 μg/ml cycloheximide (CHX) for different time points (n=1). (D) Representative Western blots of WT and TSC2KO-MEF cells left untreated or treated with 100 nM rapamycin for 1 day (WT-MEF: n=2; TSC2-KO-MEF: n=8). (E) Representative Western blots of INS-1E β-cells pre-treated with S6K1 inhibitor (10 μM) and cultured with 22.2 mM glucose for 2 days (n=3). (F) Scheme of MHY1485 action. (G) Representative Western blots of INS-1E β-cells treated with MHY1485 (25 μM) for 3 hours (n=2). (H) Scheme of 3BDO action. (I) Representative Western blots of INS-1E β-cells treated with 3BDO (10 or 20 μM) for 3 hours (n=1).

Figure S3

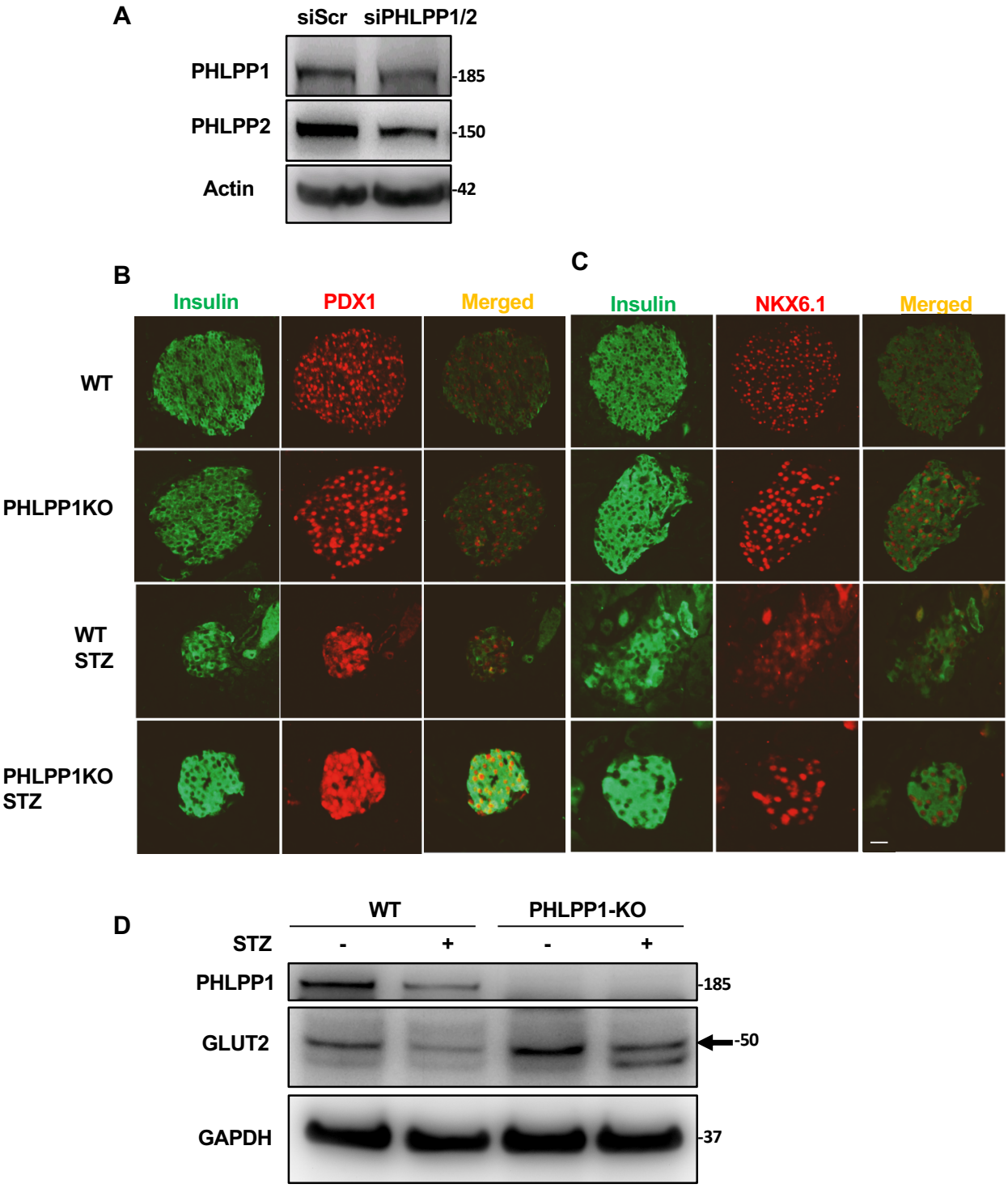

Figure S4. PHLPP1 deficiency restores PDX1, NKX6.1 and GLUT2 expression. Related to Figure 4. (A) Representative Western blot of isolated human islets transfected with PHLPP1 and PHLPP2 siRNA or control siScr for 3 days (n=4). (B,C) Representative double-stainings for PDX1 (red, B), or NKX6.1 (red, C), and insulin (green) are shown from PHLPP1-KO and WT controls mice injected with streptozotocin (STZ) or saline (n=6-7). Scale bar depicts 50µm. (D) Representative Western blot of isolated islets from PHLPP1-KO and WT control mice exposed to 1mM STZ in culture for 6h (n=2 independent experiments; each pooled from 3 mice/condition).

Figure S4

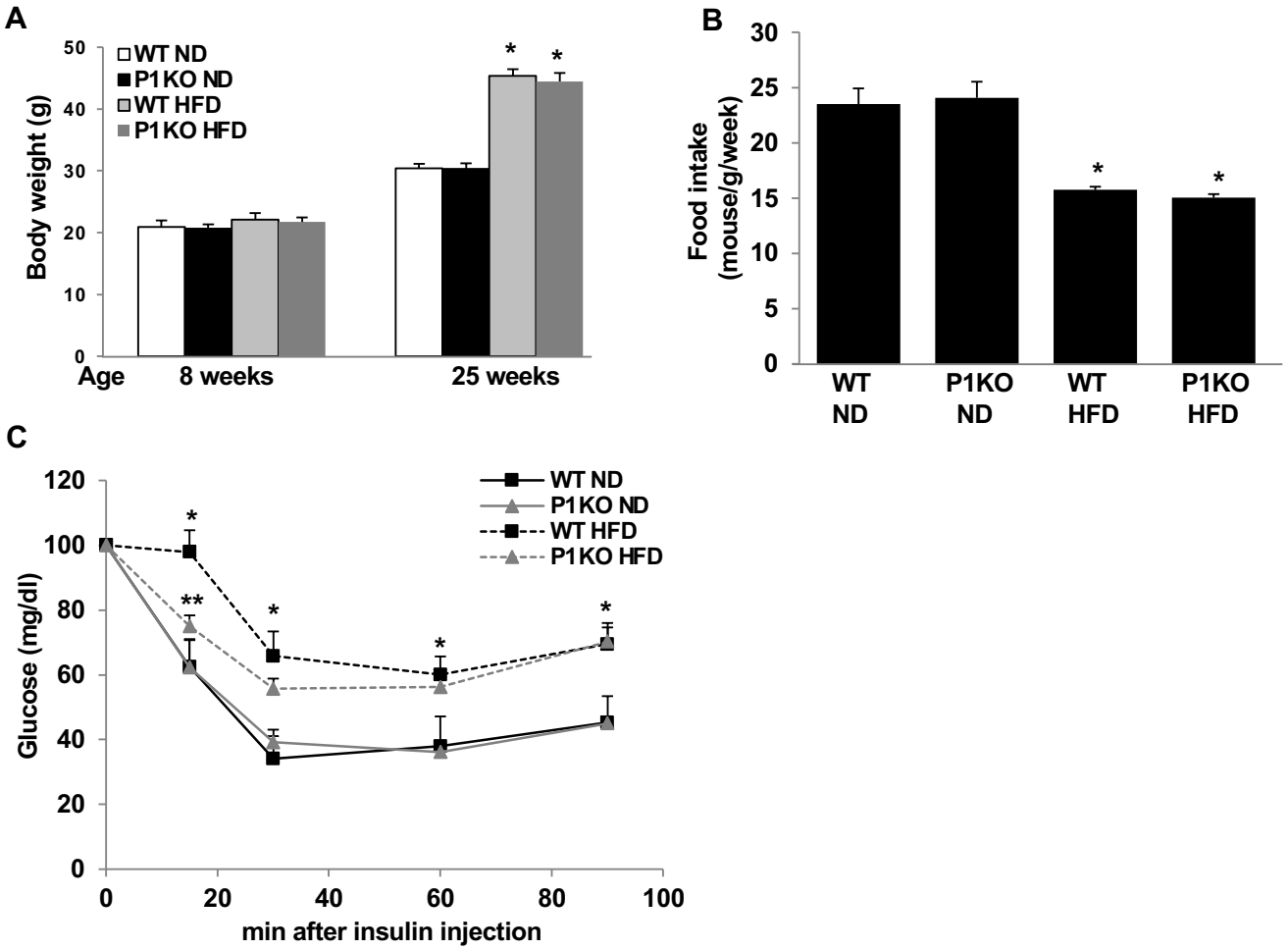

Figure S5. Characterization of ND- or HFD-fed PHLPP1-KO mice. Related to Figure 5. (A-C) PHLPP1-KO and WT controls mice were fed a normal (ND) or high fat/ high sucrose diet ("Surwit"; HFD) for 17 weeks. (A,B) Body weight and average weekly food intake/mouse (n=7-15). (C) Intraperitoneal insulin tolerance tests (ipITT) with 0.75IU/kg BW insulin (n=7-22). Data are expressed as means ± SEM. *p<0.05 WT- or PHLPP1KO-HFD compared to WT- or PHLPP1KO-ND mice. **p<0.05 PHLPP1-KO-HFD compared to WT-HFD mice.

Figure S5

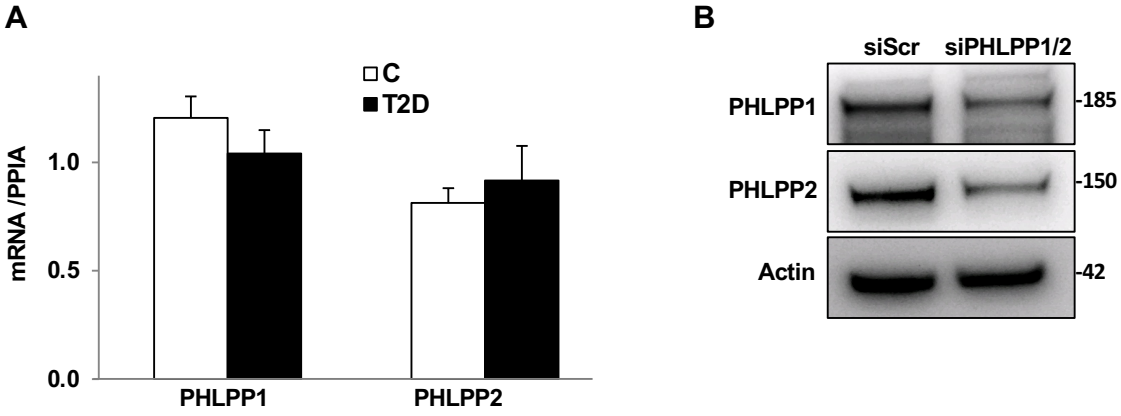

Figure S6. PHLPP1/2 expression and silencing in isolated human T2D islets. Related to Figure 6. (A) qPCR for PHLPP1 or PHLPP2 mRNA expression in human islets isolated from nondiabetic (n=24) or individuals with T2D (n=7) normalized to cyclophilin. Data are expressed as means ± SEM (B) Representative Western blots of isolated human islets from patients with T2D transfected with PHLPP1 and PHLPP2 siRNA or control siScr for 2 days (n=2).

Figure S6

Publication III

PHLPP1 deletion restores pancreatic β-cell survival and normoglycemia in the db/db mouse model of obesity-associated diabetes

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My contribution:
Designed, performed experiments, analysed data and assembled the figures for:
Figures: 1; 2 C,I,J,K,L
Wrote the paper.

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PHLPP1 deletion restores pancreatic β-cell survival and normoglycemia in the db/db mouse model of obesity-associated diabetes

Blaz Lupse[1], Nick Heise[1], Kathrin Maedler[1,3]✉ and Amin Ardestani[1,2,3]✉

The Pleckstrin homology domain leucine-rich repeat protein phosphatases (PHLPPs) are novel therapeutic targets for the restoration of β-cell survival and function in diabetes. Their upregulation and activation in β-cells under conditions of both type 1 and type 2 diabetes directly correlates with β-cell failure; β-cell death and loss of insulin secretory function through disturbance of cell survival control mechanisms. PHLPPs directly dephosphorylate and regulate activities of β-cell survival-dependent kinases AKT and MST1 constituting a regulatory triangle loop to control β-cell apoptosis. PHLPP1 deletion in severely diabetic leptin receptor-deficient db/db mice restored normoglycemia and β-cell area through increased β-cell proliferation and reduced β-cell apoptosis. The beneficial effects of PHLPP1 deficiency in a severe mouse model of obesity and diabetes make PHLPP a new target for β-cell-directed diabetes therapy.

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Type 2 diabetes (T2D) is a sophisticated metabolic disease defined by systemic insulin resistance as well as a decline in functional pancreatic β-cell mass. A progressive β-cell deterioration and associated loss in physiological insulin secretion is a central feature of T2D. β-cell function and/or mass progressively decline due to a sedentary lifestyle with abundant nutrient supply as a result of complex pathomechanisms eventually resulting in hyperglycemia and T2D [1]. Strategies to prevent β-cell failure or repair dysfunctional β-cell are urgently needed for a causative therapy targeting the cause of this severe metabolic disease.

Recently, we found the Pleckstrin homology domain leucine-rich repeat protein phosphatases 1 and 2 (PHLPP1, PHLPP2) highly upregulated in β-cells under a diabetic environment [2] and this directly correlates with β-cell death and impaired insulin secretion in human and rodent pancreatic β-cells in vitro and in vivo [2, 3]. PHLPP1/2 are members of the protein phosphatases metal-dependent (PPM) group of serine-threonine phosphatases (STPs) family and key mediators of pro-apoptotic signaling [4]. They directly dephosphorylate and inhibit pro-survival kinase AKT together with dephosphorylation and activation of pro-apoptotic MST1 [2, 4]; both pathways independently mediate β-cell failure [5, 6].

AKT serves as a main downstream executor of PI3K-IRS signaling; activated AKT boosts the mechanistic target of rapamycin complex 1 (mTORC1), which enhances growth and protein synthesis [7] and thus plays an instrumental role in the regulation of β-cell mass and function [6]. In contrast, MST1 activation results in excessive apoptosis and abolished insulin production, and is a strong mediator of diabetes progression [5]. Therefore, the PHLPP-AKT-MST1 triangle acts as a stress-sensitive survival pathway in β-cells and under physiological conditions, controls the fine balance of β-cell turnover. However, chronic overnutrition leads to sustained mTORC1 hyperactivation and subsequent PHLPP instigation which leads to imbalanced AKT/MST1 regulation and induction of β-cell apoptosis [2].

To substantiate the efficacy of genetic PHLPP1 inhibition seen in our recent study [2] in a severe in vivo mouse model of hyperglycemia and obesity-associated diabetes progression, we have deleted PHLPP1 in leptin receptor-deficient $Lepr^{db/db}$ (db/db) mice. Db/db mice become severely obese leading to β-cell dysfunction and loss, chronic hyperglycemia, and diabetes by the age of 7–12 weeks. This was seen by the rise in glucose levels at the age of 6 weeks in db/db control mice with a heterozygous PHLPP1 deletion: db/db-$PHLPP1^{+/-}$. In contrast, homozygous PHLPP1 deletion (db/db-$PHLPP1^{-/-}$) led to drastically lower glucose levels to physiological conditions during the entire 6-week study period (Fig. 1A, B) [5, 8]. While db/db control mice showed severe glucose intolerance, it was significantly improved in the db/db-$PHLPP1^{-/-}$ mice, together with a significant normalization of fasting glucose levels, indicating that PHLPP1 ablation prevents hyperglycemia (Fig. 1C, D). To test whether this metabolic improvement was due to changes in insulin sensitivity, we performed an intraperitoneal insulin tolerance test. After the insulin challenge, we did not see any differences in the ability to lower blood glucose levels in the two groups (Fig. 1E).

Despite the very low insulin sensitivity in db/db mice, the significantly elevated endogenous insulin production in db/db-$PHLPP1^{-/-}$ mice with 4-fold increased serum insulin levels compared to controls (Fig. 1F) is a trigger for the improved glucose tolerance, which suggests that the anti-hyperglycemic effect seen in the $PHLPP1^{-/-}$ mice likely comes from improved β-cell insulin secretion and/or mass. Indeed, the mice also showed a 5.7-fold increased insulin/glucose ratio at 12 weeks of age (Fig. 1G).

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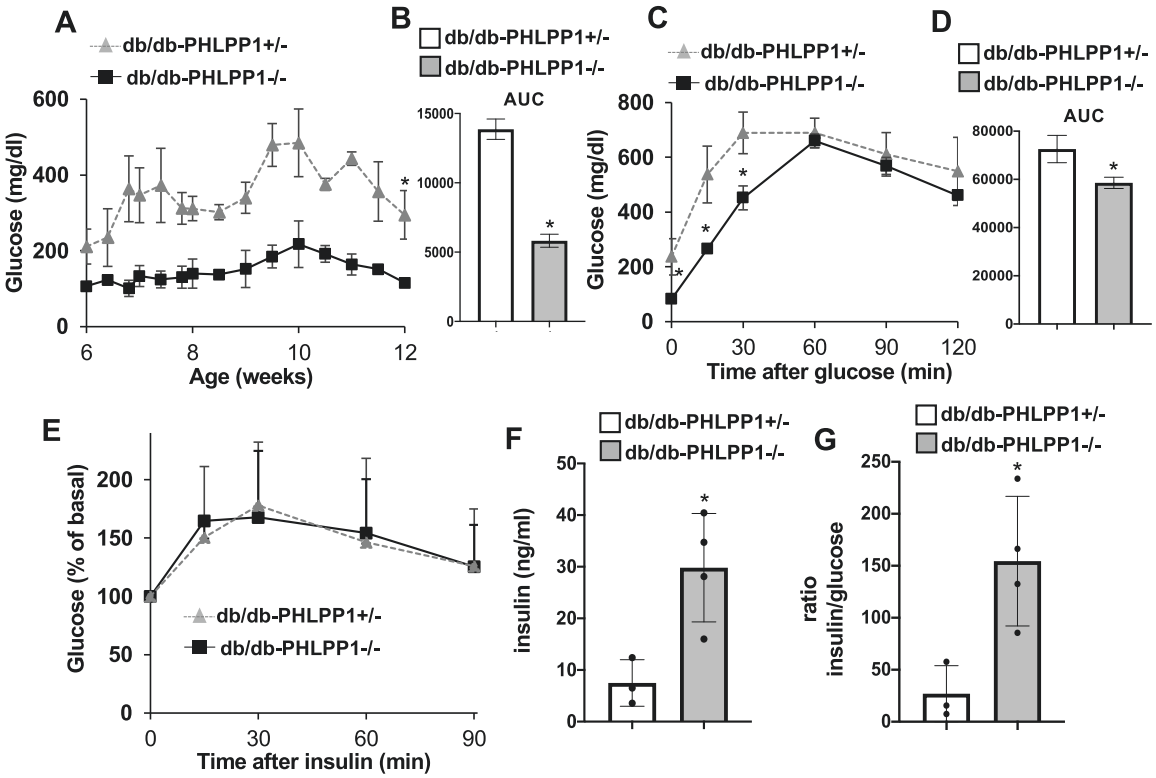


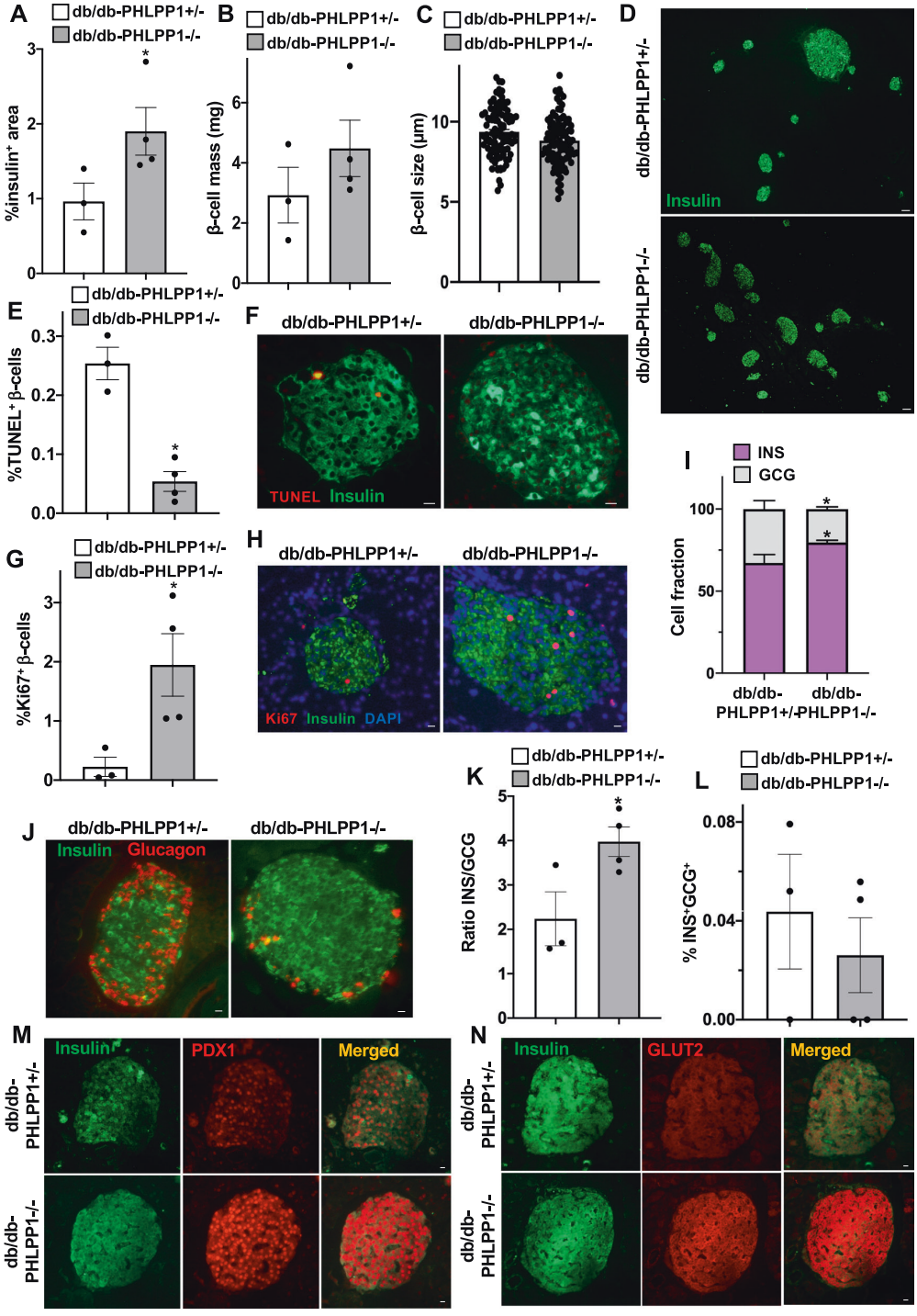
Fig. 1 PHLPP1 depletion improves glycemia in the obese db/db mouse model of type 2 diabetes. Male db/db-PHLPP1$^{-/-}$ ($n = 4$) and db/db-PHLPP1$^{+/-}$ ($n = 3$) were monitored for **A**, **B** random fed blood glucose with respective area-under-the-curve (AUC) analysis from the age of 6 weeks throughout the 6-week experiment. **C**, **D** Intraperitoneal glucose tolerance test (ipGTT) with respective AUC analysis, **E** Intraperitoneal insulin tolerance test (ipITT) with basal glucose values normalized to 100%, **F** serum insulin, and **G** the ratio of serum insulin and blood glucose calculated at fed state at the end of the study in 12-weeks-old mice. Data show means ± SEM. *$p < 0.05$ db/db-PHLPP1$^{-/-}$ compared to db/db-PHLPP1$^{+/-}$; all by Student's *t*-tests.

When we compared the heterozygous control db/db-PHLPP1$^{+/-}$ mice to WT-db/db mice from different cohorts (e.g., refs. [8–10]), glucose and insulin levels were similar, which suggests that knocking out a single PHLPP1 allele has no effect on glucose metabolism. However, one needs to keep in mind that mice are from different studies and backcrosses.

To further investigate the mechanisms of the higher insulin production in db/db-PHLPP1$^{-/-}$ mice, we dissected the pancreases and studied islet morphology and survival. Db/db-PHLPP1$^{-/-}$ showed a 2-fold significantly increased insulin-positive β-cell area, which also reflected in a tendency to increased β-cell mass with generally much larger islets, compared to controls (Fig. 2A, B, D), while β-cell size remained similar in both PHLPP1$^{-/-}$ and control mice (Fig. 2C). Such β-cell area restoration was due to an improved β-cell survival and enhanced β-cell replication. While β-cell apoptosis (shown by TUNEL-staining) was 4.7-fold reduced (Fig. 2E, F), β-cell proliferation (shown by Ki67-staining) was 8.7-fold increased (Fig. 2G, H) in db/db-PHLPP1$^{-/-}$, compared to control. Islets from db/db-PHLPP1$^{+/-}$ mice had fewer insulin-positive β-cells and an expansion of glucagon-positive α-cells, compared to db/db-PHLPP1$^{-/-}$ mice (Fig. 2I–K), which were protected against such apparent α-cell hyperplasia. While they occurred very rare, we did not observe any significant changes in bi-hormonal insulin-glucagon double-positive cells between the two groups (Fig. 2L), ruling out the possibility of trans-differentiation from α- to β-cells in db/db-PHLPP1$^{-/-}$ mice as the mechanism for the increased β-cell mass. This suggests a combined higher compensatory β-cell proliferation and a lower rate of apoptosis as a major mechanism of the β-cell mass restoration in db/db-PHLPP1$^{-/-}$ mice.

Metabolic disruption in protein networks led by chronic hyperglycemia and nutrient overload compromises the tightly regulated signaling pathways essential for β-cell identity, survival, and function. The transcription factor pancreatic duodenal homeobox-1 (PDX1) is indispensable for β-cell development and the control of glucose-stimulated insulin secretion in mature β-cells [11]. PDX1 level is declined in both human and rodent diabetic β-cells and this correlates with higher β-cell death and impaired insulin secretion [5, 12]. Correspondingly, db/db-PHLPP1$^{-/-}$ mice expressed a much higher amount of PDX1 in the nucleus compared to the control db/db-PHLPP1$^{+/-}$ counterpart (Fig. 2M). Our previous work identified PDX1 as a β-cell-specific MST1 substrate. Aberrant MST1 activity under diabetic condition promotes PDX1 degradation and inactivation through its direct phosphorylation [5]. Thus, PHLPPs as upstream activators of MST1 [2, 13] can indirectly regulate PDX1 degradation and thereby disrupt its function as a transcription factor [5]. Consequently, expression of the PDX1 downstream target GLUT2, the principal glucose transporter in rodent β-cells, which is essential for glucose sensing, was also increased in db/db-PHLPP1$^{-/-}$ mice compared to heterozygous controls (Fig. 2N).

In addition, db/db-PHLPP1$^{-/-}$ mice had a trend to lower body weight compared to the PHLPP1 heterozygous controls, however, this did not reach significance by the end of the study (data not



shown) and did not lead to an improved insulin sensitivity; the anti-hyperglycemic effect of the PHLPP1 deletion seemed to have solely come from the improved β-cell phenotype.

Our findings demonstrate beneficial effects of PHLPP1 deficiency in severely diabetic db/db mice preventing hyperglycemia as well as improving β-cell survival and insulin production in vivo.

For the development of PHLPPs as a therapeutical target, the tumor-suppressing function of PHLPPs must not be underestimated, and such was tightly monitored in our studies. Neither PHLPP1$^{-/-}$ nor db/db-PHLPP1$^{-/-}$ mice developed anatomical or physiological defects. We did not detect any changes in organ growth nor tumorigenic abnormalities in PHLPP1$^{-/-}$ mice [2],

Fig. 2 PHLPP1 depletion promotes β-cell survival in the obese db/db mouse model of type 2 diabetes. A Insulin-positive area and **B** β-cell mass analysis given as a mean percentage of the entire pancreatic section area from 10 sections/mouse throughout the whole pancreas, **C** the respective β-cell size analysis from 100 randomly chosen β-cells from each group and **D** representative insulin staining of the pancreas. **E**–**H** Quantitative analyses and representative images of **E**, **F** double-staining for TUNEL and **G**, **H** triple-staining for Ki67 both expressed as a percentage of insulin-positive β-cells (an average of 3827 and 3965 β-cells/ mouse were counted for TUNEL and Ki67 analyses, respectively for each group). Quantitative analyses (**I**, **K**) and representative images (**J**) of the percentage of glucagon-positive α-cells (red) and insulin-positive β-cells (green) together with quantitative analyses of insulin and glucagon colocalization (**L**; an average of 1455 β-cells and 529 α-cells/mouse were counted for each group). **M**, **N** Representative images for double-staining of **M** nuclear PDX1 and **N** GLUT2 expression. Data show means from all pooled analyses for each mouse ± SEM. $*p < 0.05$ db/db-PHLPP1$^{-/-}$ vs. db/db-PHLPP1$^{+/-}$ controls; all by Student's *t*-tests. Scale bars depict 50 µm in **D** and 10 µm in **F**, **H**, **J**, **M**, **N**.

possibly due to the compensatory action of other PHLPP isoform PHLPP2 [14]. PHLPPs represent a promising target to further explore mechanisms underlying the pathophysiology of the broad signaling network in diabetic islets, which potentially help to rescue pancreatic β-cells in diabetes.

DATA AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. All raw data are available upon request from the authors.

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AUTHOR CONTRIBUTIONS

Conceptualization and supervision: AA and KM; manuscript writing: BL, AA, and KM; methodology: BL, NH, AA, and KM; Formal analysis and investigation: BL, NH, and AA; funding acquisition: AA and KM.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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3 Discussion

Diabetes involves the loss of functional insulin-producing pancreatic β-cells, typically resulting from an inadequate β-cell mass. Apoptosis is considered as a hallmark for the loss of functional β-cell mass. The Hippo pathway is a prominent regulator of organ size and tissue homeostasis and critical for the regulation of β-cell function and viability. We identified that the core component of the Hippo pathway, MST1 kinase, and its upstream regulators, PHLPP phosphatases, are activated in human and rodent β-cells under multiple experimental models of diabetes and that suppression of their signals improves β-cell function and survival.

3.1 Publication I: Neratinib Protects Pancreatic β-cells in Diabetes

MST1 is a critical effector of Hippo pathway and acts as a pro-apoptotic molecule, leading to β-cell failure under diabetic conditions (1). Thus, an identification of a potent MST1 inhibitor would be a promising approach for β-cell protective therapy in diabetes. In collaboration with California Institute for Biomedical Research (CALIBR), we identified a small molecule inhibitor neratinib as a novel inhibitor of MST1. In principle, neratinib is a well-established inhibitor of ErbB family of receptor tyrosine kinases. Specifically, it targets epidermal growth factor receptor (EGFR, also known as ErbB-1) and human epidermal growth factor receptor-2 (HER2, also known as ErbB-2) (2). It is primarily used and approved by FDA in the treatment of early-stage HER2-positive breast cancer with promising clinical studies for lung, colorectal, and bladder cancers. We further showed that neratinib can successfully inhibit MST1 kinase at nM range -similar to the EGFR/HER2 inhibition- and blocks cellular MST1 activation in pancreatic β-cells/islets and protect them from apoptosis under several diabetic conditions in vitro and in vivo. Although neratinib potently blocks EGFR/HER2/MST1, it also presents some non-specific inhibition toward several other kinases as the development of a selective kinase inhibitor is highly challenging and most of commercially available kinase inhibitors have significant off-target effects (3,4). Our kinase-selectivity profiling and analysis showed that neratinib can additionally interact with other kinases from MAP4K family as well as with YSK4, GCN2, YES and others (5). Nevertheless, further studies to enhance specificity and potency toward MST1 together with minimizing its off-target effect are currently under ongoing investigation with the potential goal to reduce the gastrointestinal side effects, which are often seen with EGFR inhibitors (5). Neratinib has so far shown promising safety profile, with many clinical studies reporting low/moderate side-effects when applied alone or in combination with other anti-tumor drugs (6–8). After all, neratinib is already FDA-approved drug with well-known drug safety profile, which consequently significantly reduces the time and costs, which are usually invested in the development of a new drug.

Our work showed that neratinib treatment largely reduced MST1 phosphorylation and β-cell apoptosis under various diabetogenic conditions. We observed a significant reduction in β-cell apoptosis triggered by both glucolipotoxicity and pro-inflammatory cytokines in neratinib-treated INS-1E β-cells and isolated primary human islets as well as in isolated islets from diabetic db/db mice. Most notably, neratinib improved hyperglycemia and glucose tolerance and increased insulin secretion in both the T1D (MLD-STZ) and the T2D (db/db) mouse models. Neratinib not only presented a robust anti-apoptotic response but also restored β-cell maturation and function as represented by significant restoration of important β-cell markers for glucose metabolism and insulin production (PDX1, NKX6.1, and GLUT2) in the diabetic mice. As already mentioned in the introduction (chapter 1.4), other MST1 inhibitors (e.g., LP-945706 (9) and XMU-MP-1 (10)) have been introduced as well and both already tested in vivo. Very similar to our study, Faizah *et al.* administered XMU-MP-1 (XMU) *in vivo* in STZ-treated mice for 21 consecutive days. By using the STZ-induced diabetic mouse model, they observed a strong improvement in glucose tolerance as well as an increase in β-cell number and β-cell area in the XMU-treated group compared to the control diabetic group (10). Moreover, XMU additionally showed clear benefits in cellular repair and regeneration in intestine and liver (11). Based on these data we might assume that relatively short treatment period (20-30 days) with MST1 inhibitor might be sufficient for the therapy in patients with diabetes. In contrary, little is known about pharmaceutical inhibition of another main Hippo kinases LATS1/2, whose hyperactivation has similar pro-apoptotic and pro-diabetic impacts (12). Nevertheless, very recent studies demonstrated successful LATS1/2 inhibition by two newly discovered LATS1/2 inhibitors (13,14).

Recent investigations demonstrate the role of HER2/EGFR, neratinib targets, beyond oncogenesis. Serum HER2 levels are significantly elevated in subjects with hyperglycemia and insulin resistance and decrease after weight loss (15,16). Diabetic complications, such as cardiovascular disease and nephropathy, are also associated with EGFR signals (17). In line with this, EGFR inhibitor erlotinib was able to decrease the fasting glucose levels in two independent lung cancer patients with T2D (18,19). Likewise, the treatment with similar tyrosine kinase inhibitors (TKI) such as imatinib (inhibit c-Abl and PDGFR), and sunitinib (inhibit PDGFR and VEGFR2) also improved hyperglycemia and reverse diabetes progression (20). Moreover, in vivo data of imatinib in diabetic rodent models have shown improvement in insulin resistance and remission of diabetes by inhibiting the production of tumor necrosis factor alpha (TNF-α) and by reducing ER stress (21,22). One possible link connecting tyrosine kinase signaling to diabetes can be through fatty acid synthase (FASN) activity, which has been linked to insulin-resistant conditions such as obesity and T2D (23). HER2 has been closely related to enzymes associated with lipid metabolism (including FASN), PI3K, and

mTOR (24), and it strongly correlates with increased expression of FASN (25). Moreover, higher circulating HER2 levels are consistent with increased incidence of diabetes in a large population-based study (17). The relationship between high HER2 and glucose levels was also observed in newly diagnosed T2D patients (26). Interestingly, HER2 levels can be downregulated using established first-line treatment for diabetes, metformin (27). It is therefore plausible that extra-pancreatic effect of neratinib- in particular targeting HER2/EGFR signaling in peripheral metabolic organs like liver and fat- might contribute to the anti-diabetic effect of neratinib. Our lab further reported a clinical case where combined HER2/EGFR/MST1-inhibition by neratinib effectively restores normoglycemia in a patient with HER2 positive breast cancer and T2D comorbidity during 18-month treatment period, suggesting neratinib as a promising dual-target therapeutic approach for the cancer-diabetes comorbidity (28).

EGFR signaling and effect of its inhibition on pancreatic β-cells survival and function remains largely unknown. As described, EGFR is crucial in the development of pancreatic islets and for their proliferation. Inhibition of EGFR signaling in islets during pregnancy or in the first weeks after birth leads to diabetes (29,30). Fewer known effects are reported in mature islets. To exclude the effect of EGFR inhibition of neratinib in β-cells, we used closely related EGFR inhibitor canertinib that lacks MST1 inhibitory activity. Neratinib but not canertinib significantly reduced induction of apoptosis in INS-1E β-cells under diabetic condition. Neratinib largely prevented stress-induced MST1 activation and apoptosis, while the EGFR inhibitor canertinib stayed ineffective. These results suggest that the beneficial action of neratinib is likely independent of EGFR inhibition. Yet, we cannot completely exclude off-target effects nor definitively declare MST1 as the only target of neratinib. One possible approach to prove an MST1-specific effect would be CRISPR-mediated deletion of all other potential neratinib-sensitive kinases (31). Nevertheless, based on our work and other studies which report detrimental effects of MST1 in β-cells (1,10,32), we believe that the underlying mechanisms of strong diabetes protection still derives from direct inhibition of MST1 rather than from inhibition of other kinases. Such pharmacological inhibition of MST1 is also strongly supported by the genetic MST1 deficiency in MST1-KO mice, which leads to favorable effects in terms of glycemic normalization and improvement in β-cell function and survival (1). Furthermore, under diabetogenic conditions, neratinib reduces apoptosis in islets isolated from WT mice but exerts no additive effect in MST1-KO islets, assuming that MST1 inhibition by neratinib is thus sufficient to restore β-cell survival. MST1/2 has a crucial role in the regulation of the apoptotic pathways, but it also inhibits autophagic flux, a protective mechanism in β-cell against nutritional and metabolic stress. In heart, activated MST1 phosphorylates autophagy regulator Beclin1 at T108 residue and directly inhibits the Beclin1-Vps34 complex, which suppresses

the autophagy and promote cardiomyocyte death (33–35). Therefore, MST1 inhibition by neratinib might have dual beneficial effects by boosting pro-survival autophagy response and reducing apoptosis pathway in β-cells.

Disturbance in the MST1 expression or activity can reduce cell apoptosis and excessively initiates YAP-dependent proliferative programs, with a high potential to activate oncogenic cell signaling and uncontrolled growth. Therefore, MST1 activation should always be strictly regulated in order to limit YAP-mediated uncontrolled cell proliferation and cell expansion. So far, no drugs targeting the MST1 kinase have been approved for clinical use. Since proliferation in mature human islets is negligible due to cellular quiescence and minimal activity of proliferative signaling i.e., loss of YAP expression in the mature endocrine cells, it would be practical to limit MST1 inhibition to pancreatic islets. In such way, we would avoid possible tumor development in other tissues with much higher YAP expression and allow β-cells to enhance their survival and function. If the usage of neratinib or any other kinase inhibitors is chronic for a longer period, careful toxicological studies are necessary to ensure their safety and efficiency of such inhibitors, which are often unspecific.

3.2 Publication II and III: Inhibition of PHLPP1/2 Phosphatases Rescues Pancreatic β-cells in Diabetes

In a subsequent project of my thesis, I could directly link MST1 with PHLPP phosphatases' upregulation in diabetic β-cell. As described in the introduction (Chapter 6) repression or aberrant hyperactivation of PHLPP contributes to several serious diseases, including cancer, cardiovascular disorders and diabetes, and PHLPPs are key regulators of cell apoptosis. (36–40). We provided extensive evidence and insight that PHLPPs levels were highly elevated in metabolically stressed rodent and human diabetic pancreatic islets and their forced up-regulation induces β-cell failure. PHLPPs directly dephosphorylate and regulate activities of two important kinases for β-cell survival/apoptosis namely AKT and MST1 forming a regulatory triangle loop to control β-cell viability and stress responses. Genetic inhibition of PHLPPs improves β-cell survival and function in experimental models of diabetes *in vitro*, *in vivo* and in primary human islets isolated from T2D donors. In addition, we showed that prolonged mTORC1 activation is the upstream regulator of PHLPPs upregulation connecting metabolic stress to β-cell death. Mechanistically, PHLPPs protein translation is controlled by mTORC1 signaling and its inhibition either pharmacologically or genetically reduces PHLPPs protein levels under glucotoxic condition in pancreatic β-cells.

My work demonstrates up- and down-stream signal transductions of PHLPPs that are activated by the pro-diabetic conditions in pancreatic β-cells. They interact with important metabolic pathways, such as Hippo and AKT-mTORC1 pathways, and regulate β-cell survival. After Gao et al. initially showed that PHLPPs are able to interact with AKT and dephosphorylates it at S473, suppressing its activation (41), multiple studies including ours confirmed this original observation in the context of insulin resistance and diabetes in heart, liver, adipose tissue and pancreatic islets (36,42–46). Qiao *et al.* then identified MST1 as a novel substrate for PHLPPs. When dephosphorylated at T387 by PHLPPs, MST1 auto-phosphorylation at T183 is enhanced, which subsequently leads to MST1 activation and cell death (47). We further demonstrated that MST1 is an important target of PHLPPs in pancreatic β-cells and its dephosphorylation by PHLPP controls β-cell death under diabetic settings. Moreover, MST1 also physically interacts with AKT and is its direct inhibitor (48). Both mechanisms, PHLPP-MST1 as well as MST1-AKT, cumulatively amplify pro-apoptotic MST1 signaling. In turn, activated AKT can directly interact and phosphorylate MST1, preventing its apoptosis-promoting effect (49,50). Under normal state, PHLPPs simultaneously activate and inactivate AKT and MST1 respectively, ensuring a balance for β-cell survival and apoptosis. However, diabetes induced PHLPPs up-regulation triggers higher activation of MST1 and inactivation of AKT, leading to β-cell dysfunction and death. To further substantiate anti-apoptotic response of PHLPP deficiency *in vivo*, we thoroughly investigated PHLPP1-KO mice in three different diabetic mouse models: a) STZ-induced β-cell destruction and diabetes; b) HFD-induced obesity, β-cell failure and diabetes and c) Leptin receptor-deficient obese db/db mice with profound loss of β-cell function and mass. PHLPP1 deletion effectively mitigates diabetes progression in all three models of diabetes with enhanced β-cell mass and proliferation, reduced apoptosis, and restored insulin secretion and glucose tolerance. In line with our data, others also reported beneficial effects of PHLPP1 silencing in additional pathological settings. Hypothalamic silencing of PHLPP1 leads to improved hypothalamic insulin and leptin signaling and thus greater weight loss and reduction in adiposity with improved insulin signaling in obese mice (51). Furthermore, PHLPP1 deletion in the heart is reported to be cardioprotective by enhancing pro-survival AKT activity in cardiac myocytes and increases its survival during ischemia (52). PHLPP1 depletion is also neuroprotective by increasing AKT signaling in the brain during ischemic insult (53). Importantly, deletion of both PHLPP isoforms may not be necessary to achieve beneficial effects and is even non-preferred with potentially oncogenic outcome. Besides, specific PHLPP isoforms selectivity inactivate specific AKT isoforms. As reported PHLPP1 plays a greater role in glucose homeostasis (where AKT2 is critical) whereas PHLPP2 has more effect on cell survival (where AKT1 is

critical) (54). This suggests that the effect of different PHLPP isoforms is tissue dependent and might even vary within cellular types.

mTORC1 signaling is a classical pathway for nutrient sensing and anabolic growth. Liu *et al*. showed that pharmacological inhibition (by rapamycin) or genetic knockdown of mTOR decreases PHLPPs expression, while the rapamycin-insensitive p70S6K mutant shows increased expression of PHLPPs (55). We confirmed these results in a β-cell line, and human islets as well as in pancreatic islets isolated from HFD-fed mice, all treated with rapamycin to inhibit mTORC1 activity. In addition, genetic inhibition of the main component of mTORC1, Raptor, by siRNA in INS-1E β-cells and human islets isolated from donors with T2D also drastically reduces PHLPPs levels. In contrast, forced activation of mTORC1 signaling by small molecules MHY1485 and 3-BDO increases PHLPPs expression in β-cells. We further showed that chronic high glucose induced PHLPP induction neither occurred at transcriptional nor post-translational levels. Therefore, we suspected that such regulation might occur at translational level. Interestingly, the 5‘ untranslated region (UTR) of PHLPP1 and PHLPP2 mRNAs contain TOP and TOP-like motifs, respectively, which are known to be controlled by mTORC1 at translation level indicating a potential mTORC1-dependent regulation of PHLPPs. To test this, we isolated active polyribosomes including associated RNAs and nascent peptides. Islets/ β-cells under metabolic stress display a higher rate of PHLPPs translation. These data indicate that PHLPP is an important factor of chronic mTORC1 activation and that mTORC1 is required for the detrimental action of PHLPPs in stressed pancreatic β-cells.

Importantly, as shown previously by us, mTORC1 is hyperactivated in diabetic mouse models and human islets isolated from donors with T2D (56). Activated mTORC1 potentially enhances the inhibition of pro-survival AKT signaling in diabetic β-cells via its negative feedback loops (57). Since mTORC1 controls PHLPPs and PHLPPs target AKT, mTORC1-PHLPP axis would be additional loop which could suppress AKT and initiates apoptosis. This suggests that chronic nutrient excess and subsequent hyperactivation of mTORC1 initiates the PHLPP mediated negative feedback loop, among many others, which aim to rebalance highly induced mTORC1 by terminating mTORC1 upstream regulators including AKT. In principle, such loop is in place as a natural regulatory mechanism to fine-tune cellular metabolism, i.e., a safety mechanism by which mTORC1 suppresses its own oncogenic potential. However, excessive signals triggered by mTORC1 controlled loops deplete AKT and its downstream targets and eventually leads to cellular collapse and death.

Although loss of PHLPPs could be a promising tool to protect β-cells in diabetes, the tumor-suppressing function of PHLPPs is required to ensure a tight control of oncogenic pathways

and to block tumorigenesis (58–60). As such, systemic PHLPP1 deletion and its general impact was carefully monitored in our studies. Notably, PHLPP1-KO mice do not show any obvious anatomical or physiological defects. PHLPP1-KO mice are viable, fertile, and show no significant differences in food intake and body weight compared to WT control mice. We did not detect any changes in organ over-growth or tumorigenic abnormalities in PHLPP1-KO mice, possibly due to the compensatory effect of PHLPP2 isoform or other AKT signaling phosphatase PTEN (61). In line with the anti-diabetic effect of PHLPP1 inhibition documented in our studies, mice with global or local deletion/inhibition of PHLPP1 show remarkable neuro-, cardio-, and intestine-protection as well as tissue regeneration in multiple disease models (62–65). Signaling components of regenerative and oncogenic pathways are generally very similar, differing mostly in their intensity and the duration of activation and in their safety mechanisms to strictly control their „on“ and „off“ state. Pharmacological inhibition of PHLPPs is therefore limited due to their expanding roles in controlling diverse, and often opposing, signaling pathways. Optimally designed drugs targeting PHLPPs would need to be selective with relative (not absolute as seen in PHLPP1$^{-/-}$ model) PHLPPs inhibition restricted to the specific tissue or cell type. Such therapy would have great potential, which is unlikely to lead to uncontrolled cell proliferation and tumor development. So far, several small-molecule inhibitors of PHLPPs have been developed (66); we have tested two of them (NSC117079 and NSC45586) (67) in our preliminary experiments in cell line and primary islets. However, lack of selectivity, moderate cell toxicity and poor in vivo applicability precluded their further analyses. In contrary, some studies reported successful inhibition of PHLPPs and AKT activation using those two inhibitors in different cell/tissue types and pathological settings (67–69). Importantly, as reported NSC45586 inhibitor binds to serum albumin, which might largely limit its *in vivo* use (69).

Outlook

The studies I have conducted for my thesis provide significant gain in knowledge concerning the understanding of the pathophysiology in pancreatic β-cell and signaling pathways leading to β-cell death and diabetes. In addition to the great number of answers delivered herein, the studies generated new questions and concerns, which raise the need for future investigations:

A) Inhibition of MST1 by neratinib is sufficient to restore β-cell function and survival under diabetic conditions. However, neratinib as well as other kinase inhibitors, target many other kinases since the development of specific kinase inhibitors is very challenging. Optimization of neratinib-based MST1 inhibitors that show improved potency and selectivity towards MST1 with minimum off-targets and a great safety profile is necessary for the treatment of chronic diabetes. Such optimizations are currently in progress.

B) PHLPPs are emerging class of phosphatases which tightly regulate proliferation and apoptosis at cellular and systemic levels. While new discoveries are constantly being made about the role of PHLPP isoforms, there are still plenty details unknown about how PHLPPs contribute to the pathophysiology of diseases such as diabetes, so better understanding of their signaling is necessary.

C) Development of a safe and potent PHLPP small molecule inhibitor would be an encouraging option but only in cases, where suppressing PHLPP would have mutual beneficial effects for various diseases. For example, cancers driven by oncogenes that suppress PKC (70) would benefit from restoring PKC by inhibiting PHLPP function. In such case PHLPP inhibitors could improve cancer outcome and associated diabetes or vice versa. Very similar case has been studied recently in the regard of neratinib (28). Certainly, most optimal would be the development of a small molecule inhibitors selective for β-cells, which might be a future therapeutic tool in the treatment of diabetes, but such optimization would be particularly challenging.

D) β-cell specific deletion/inhibition of PHLPPs would be the most promising choice to prevent PHLPP-mediated diabetes progression with minimal systemic side targets and side effect. For example, using nucleic acid inhibiting PHLPPs at the RNA level (i.e., antisense oligonucleotides) conjugated with β-cell specific molecules (e.g., GLP1) would be highly specific and effective (same can be extrapolated to MST1 inhibition). Such development and optimizations are under way.

Conclusions

In my studies I put my major efforts to identify molecular events and cellular mechanisms that are responsible for β-cell failure in diabetes, with identification of potent strategies to prevent β-cell death and consequent diabetes. I showed that:

- neratinib serves as a β-cell-protective MST1 kinase inhibitor improving β-cell survival and function under diabetogenic conditions. Neratinib effectively prevents apoptosis in human islets and restore normoglycemia in T1D and T2D rodent models.
- PHLPP1/2 levels are highly elevated in diabetic mouse models and in human islets exposed to diabetogenic conditions *in vitro* and its up-regulation leads to β-cell failure in diabetes.
- PHLPPs negatively regulate pro-survival AKT and activate pro-apoptotic MST1 signaling in pancreatic β-cells, forming a stress-sensitive cellular loop to control β-cell viability.
- sustained mTORC1 hyper-activation is the important mechanism of PHLPPs' upregulation in the β-cells under glucotoxicity.
- genetic inhibition of PHLPPs markedly improves β-cell survival and function and prevents diabetes progression in experimental models of diabetes *in vitro*, *ex vivo* in primary human T2D islets and *in vivo* in two animal models of T1D and T2D.

Based on data presented in this thesis and data from other studies, a positive answer can be given to the question, whether β-cell failure can be prevented or reversed in T2D. Although additional studies and/or optimizations are still needed, I believe we are on the right (Hippo) path.

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Appendices

A) Contributions in Research Articles

The Hippo kinase LATS2 impairs pancreatic β-cell survival in diabetes through the mTORC1- autophagy axis (Research Article)

Ting Yuan, Karthika Annamalai, Shruti Naik, Blaz Lupse, Shirin Geravandi, Anasua Pal, Aleksandra Dobrowolski, Jaee Ghawali, Marina Ruhlandt, Kanaka Durga Devi Gorrepati, Zahra Azizi, Dae-Sik Lim, Kathrin Maedler & Amin Ardestani

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My contribution:
Performed experiments and analysed data for Figures 1 (D,E - partially),F,G; 9 and Suppl. Figure 1 D,E; 5 B

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ARTICLE

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The Hippo kinase LATS2 impairs pancreatic β-cell survival in diabetes through the mTORC1-autophagy axis

Ting Yuan[1,4,5], Karthika Annamalai[1,5], Shruti Naik[1], Blaz Lupse[1], Shirin Geravandi[1], Anasua Pal[1], Aleksandra Dobrowolski[1], Jaee Ghawali[1], Marina Ruhlandt[1], Kanaka Durga Devi Gorrepati[1], Zahra Azizi[1,2], Dae-Sik Lim[3], Kathrin Maedler[1,6]✉ & Amin Ardestani[1,2,6]✉

Diabetes results from a decline in functional pancreatic β-cells, but the molecular mechanisms underlying the pathological β-cell failure are poorly understood. Here we report that large-tumor suppressor 2 (LATS2), a core component of the Hippo signaling pathway, is activated under diabetic conditions and induces β-cell apoptosis and impaired function. LATS2 deficiency in β-cells and primary isolated human islets as well as β-cell specific LATS2 ablation in mice improves β-cell viability, insulin secretion and β-cell mass and ameliorates diabetes development. LATS2 activates mechanistic target of rapamycin complex 1 (mTORC1), a physiological suppressor of autophagy, in β-cells and genetic and pharmacological inhibition of mTORC1 counteracts the pro-apoptotic action of activated LATS2. We further show a direct interplay between Hippo and autophagy, in which LATS2 is an autophagy substrate. On the other hand, LATS2 regulates β-cell apoptosis triggered by impaired autophagy suggesting an existence of a stress-sensitive multicomponent cellular loop coordinating β-cell compensation and survival. Our data reveal an important role for LATS2 in pancreatic β-cell turnover and suggest LATS2 as a potential therapeutic target to improve pancreatic β-cell survival and function in diabetes.

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LDHA is enriched in human islet alpha cells and upregulated in type 2 diabetes

Paulina Karen Mendoza Sanchez, Mona Khazaei, Eva Gatineau, Shirin Geravandi, Blaz Lupse, Huan Liu, Ralf Dringen, Anne Wojtusciszyn, Patrick Gilon, Kathrin Maedler and Amin Ardestani

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LDHA is enriched in human islet alpha cells and upregulated in type 2 diabetes

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ABSTRACT

The lactate dehydrogenase isoform A (LDHA) is a key metabolic enzyme that preferentially catalyzes the conversion of pyruvate to lactate. Whereas LDHA is highly expressed in many tissues, its expression is turned off in the differentiated adult β-cell within the pancreatic islets. The repression of LDHA under normal physiological condition and its inappropriate upregulation under a diabetogenic environment is well-documented in rodent islets/β-cells but little is known about LDHA expression in human islet cells and whether its abundance is altered under diabetic conditions. Analysis of public single-cell RNA-seq (sc-RNA seq) data as well as cell type-specific immunolabeling of human pancreatic islets showed that LDHA was mainly localized in human α-cells while it is expressed at a very low level in β-cells. Furthermore, LDHA, both at mRNA and protein, as well as lactate production is upregulated in human pancreatic islets exposed to chronic high glucose treatment. Microscopic analysis of stressed human islets and autopsy pancreases from individuals with type 2 diabetes (T2D) showed LDHA upregulation mainly in human α-cells. Pharmacological inhibition of LDHA in isolated human islets enhanced insulin secretion under physiological conditions but did not significantly correct the deregulated secretion of insulin or glucagon under diabetic conditions.

1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder closely linked to multiple genetic and environmental factors which together evoke the development of multiple pathophysiological metabolic disturbances. T2D is a bi-hormonal disorder manifested by a relative hypoinsulinaemia and hyperglucagonaemia leading eventually to hyperglycemia and diabetes and its complications [1]. Deregulated secretion of both hormones insulin and glucagon produced by pancreatic β- and α-cells respectively is a characteristic feature of T2D [1–5]. The interplay between these two hormones and their respective receptors located in the liver, muscle and adipose tissue enables the maintenance of glucose homeostasis, which is achieved via several mechanisms participating in the fine-tuning of insulin secretion [1]. Insulin secretory function of β-cells is defective in T2D with a higher basal release of insulin in fasting periods and insufficient insulin release after a meal [6]; the secretory defect of β-cells is caused by multiple factors, including chronically elevated glucose ("glucotoxicity") [7].

A key aspect of β-cell biology is the tight coupling between cellular metabolism and insulin secretion in order to maintain systemic energy homeostasis. To achieve this, islet cells and specifically β-cells show selective repression of some key metabolic

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B) Contributions in Review Articles

mTORC1 signaling: a double-edged sword in diabetic β-cells

Amin Ardestani, Blaz Lupse, Yoshiaki Kido, Gil Leibowitz, and Kathrin Maedler

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My contribution:
Preparation of Figures
Drafting of the manuscript: 20%

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Cell Metabolism
Review

mTORC1 Signaling: A Double-Edged Sword in Diabetic β Cells

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The mechanistic target of rapamycin complex 1 (mTORC1) is a central regulator of metabolic and nutrient cues that integrates environmental inputs into downstream signaling pathways to control cellular metabolism, growth, and survival. While numerous *in vitro* and *in vivo* studies reported the positive functions of mTORC1 in the regulation of β cell survival and proliferation under physiological conditions, more recent work demonstrates the opposite in the long term; this is exemplified by the constitutive inappropriate hyper-activation of mTORC1 in diabetic islets or β cells under conditions of increased β cell stress and metabolic demands. These recent findings uncover mTORC1's importance as an emerging significant player in the development and progression of β cell failure in type 2 diabetes and suggest that mTORC1 may act as a "double edge sword" in the regulation of β cell mass and function in response to metabolic stress such as nutrient overload and insulin resistance.

Introduction

The prevalence of obesity and diabetes increases dramatically worldwide; unfortunately, current health care systems fail to prevent these metabolic diseases and their complications. Over 420 million people have diabetes, and it is predicted that the number of affected patients who suffer from obesity-associated type 2 diabetes (T2D) will rise even further in the next decade (WHO, 2017). Pancreatic β cell failure (loss of β cell function and mass) is a central hallmark of T2D. Longitudinal prospective studies of patients with T2D exhibit a gradual decline in β cell function (Festa et al., 2006). Also, individuals with T2D show progressive β cell deterioration along with worsening of glycemia over time (U.K. Prospective Diabetes Study Group, 1995; Kahn et al., 2006; Matthews et al., 1998; Turner et al., 1996). All these highlight the urgent need for better understanding of the mechanisms underlying β cell dysfunction in diabetes and for developing novel therapeutic approaches to halt β cell failure in T2D. Although not fully understood, several mechanisms for the loss of functional β cells in T2D have been proposed, including apoptosis (Ardestani et al., 2014; Butler et al., 2003; Mathis et al., 2001; Rhodes, 2005), β cell dedifferentiation (Cinti et al., 2016; Jeffery and Harries, 2016; Talchai et al., 2012), and failure of adaptive expansion due to impaired proliferation (Tiwari et al., 2016).

Chronic exposure of β cells to nutrient overload (glucose and free fatty acids, or FFAs) triggered by a carbohydrate- and fat-rich diet is a major cause of β cell dysfunction in T2D (Alejandro et al., 2015; Leibowitz et al., 2010; Muoio and Newgard, 2008; Prentki and Nolan, 2006; Robertson et al., 2004). In addition to glucose and FFAs, recent studies suggest that blood levels of amino acids, especially branched-chain amino acids (BCAAs; leucine, isoleucine and valine) and their related metabolites, are chronically elevated in insulin-resistant states and obesity (Newgard et al., 2009). BCAA metabolism could lead to the accumulation of toxic metabolites that cause β cell mitochondrial dysfunction and apoptosis in T2D (Lynch and Adams, 2014). Conversely, feeding rodents a low-BCAA diet improves the metabolic state in mice (Fontana et al., 2016) and muscle insulin sensitivity in diabetic rats (White et al., 2016). While nutrients are needed as an energy source for all cells, their excess might lead to oxidative stress, endoplasmic reticulum (ER) stress, and inflammation, which might eventually cause β cell apoptosis (Alejandro et al., 2015; Donath et al., 2013; Leibowitz et al., 2010; Muoio and Newgard, 2008; Prentki and Nolan, 2006; Robertson et al., 2004). Initially, β cells activate defense mechanisms aimed to alleviate stress; however, constitutive activation of these pathways might eventually exacerbate β cell dysfunction and diabetes. Too much of the good thing was initially described by Paracelsus in 1538 (Paracelsus, 1538) as "sola dosis facit venenum"—that nothing is without poison: whether something becomes poisonous is determined by the dosage. As an example, nutrients have an important role in stimulating β cell proliferation and insulin secretion; however, prolonged nutrient overload shifts β cell compensation into decompensation (Weir and Bonner-Weir, 2004).

Metabolic pathways and related molecular mechanisms responsible for β cells' damage during over-nutrition are not clearly defined. The mechanistic target of rapamycin complex 1 (mTORC1) plays a key role in nutrient sensing and integration of metabolic, energy, hormonal, and nutritional stimuli to regulate cellular metabolism, survival and growth (Laplante and Sabatini, 2012). While physiological mTORC1 activation is essential for the maintenance of β cell homeostasis, adaptation, insulin secretion, and development (Blandino-Rosano et al., 2012; Kulkarni et al.,

mTORC2 signaling: a path for pancreatic β-cell's growth and function

Ting Yuan, Blaz Lupse, Kathrin Maedler and Amin Ardestani

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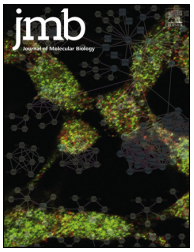

mTORC2 Signaling: A Path for Pancreatic β Cell's Growth and Function

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Abstract

The mechanistic target of rapamycin (mTOR) signaling pathway is an evolutionary conserved pathway that senses signals from nutrients and growth factors to regulate cell growth, metabolism and survival. mTOR acts in two biochemically and functionally distinct complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2), which differ in terms of regulatory mechanisms, substrate specificity and functional outputs. While mTORC1 signaling has been extensively studied in islet/β-cell biology, recent findings demonstrate a distinct role for mTORC2 in the regulation of pancreatic β-cell function and mass. mTORC2, a key component of the growth factor receptor signaling, is declined in β cells under diabetogenic conditions and in pancreatic islets from patients with type 2 diabetes. β cell-selective mTORC2 inactivation leads to glucose intolerance and acceleration of diabetes as a result of reduced β-cell mass, proliferation and impaired glucose-stimulated insulin secretion. Thereby, many mTORC2 targets, such as AKT, PKC, FOXO1, MST1 and cell cycle regulators, play an important role in β-cell survival and function. This indicates mTORC2 as important pathway for the maintenance of β-cell homeostasis, particularly to sustain proper β-cell compensatory response in the presence of nutrient overload and metabolic demand. This review summarizes recent emerging advances on the contribution of mTORC2 and its associated signaling on the regulation of glucose metabolism and functional β-cell mass under physiological and pathophysiological conditions in type 2 diabetes.

Introduction

Diabetes is a chronic metabolic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively respond to insulin. Type 2 diabetes (T2D) is characterized by insulin resistance (failure of key metabolic tissues to properly respond to insulin) as well as progressive decline in insulin secretory function and reduced pancreatic β-cell mass, resulting in the development of a relative insulin deficiency [1,2]. Sensing of glucose and other nutrients by the β cell is the initial step toward glucose metabolism to finally result in a perfectly adjusted pulsatile secretion of insulin to maintain euglycemia [3]. In obesity—associated with insulin resistance—an enhanced insulin response to nutrients is required; such metabolic adaptation is mediated by an increased β-cell number and function. The enormous plasticity and capacity of healthy β cells to adapt to such metabolic pressure is instrumental for maintenance of glucose homeostasis and prevention of T2D. Compensatory failure of the β cell under genetic and environmental influence leads to the development of T2D. In fact, β-cell failure has been described as the primary determinant of the transition from obese, insulin-resistant state to T2D [4–6]. While short-term nutrient stimulations are physiologically important for the regulation of β-cell mass and function, β cells in T2D are chronically exposed to elevated concentrations of nutrients including glucose, amino acids and free fatty acids (FFAs) which may lead to a progressive loss of β-cell function and survival by induction of a plethora of deleterious mechanisms such as oxidative stress, endoplasmic reticulum (ER) stress and inflammation leading to ultimate reduction of a functional β-cell mass in diabetes [4–10].

The signal transduction pathways and regulatory mechanisms of how nutrients and growth factors

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Hippo signaling: key emerging pathway in cellular and whole-body metabolism

Amin Ardestani, Blaz Lupse, and Kathrin Maedler

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Trends in Endocrinology & Metabolism

CellPress
REVIEWS

Review

Hippo Signaling: Key Emerging Pathway in Cellular and Whole-Body Metabolism

Amin Ardestani,[1,*] Blaz Lupse,[1] and Kathrin Maedler[1,*]

The evolutionarily conserved Hippo pathway is a key regulator of organ size and tissue homeostasis. Its dysregulation is linked to multiple pathological disorders. In addition to regulating development and growth, recent studies show that Hippo pathway components such as MST1/2 and LATS1/2 kinases, as well as YAP/TAZ transcriptional coactivators, are regulated by metabolic pathways and that the Hippo pathway controls metabolic processes at the cellular and organismal levels in physiological and metabolic disease states such as obesity, type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD), cardiovascular disorders, and cancer. In this review we summarize the connection between key Hippo components and metabolism, and how this interplay regulates cellular metabolism and metabolic pathways. The emerging function of Hippo in the regulation of metabolic homeostasis under physiological and pathological conditions is highlighted.

Highlights

Hippo pathway core components are regulated by metabolic processes and nutrient-sensing pathways.

The Hippo pathway controls lipid and glucose metabolism at both the cellular and organ levels.

Several key members of the Hippo pathway, such as MST, LATS, YAP, and TAZ, are important regulators of adipocyte proliferation and differentiation.

The Hippo pathway regulates cardiac cell homeostasis including cardiomyocyte survival, proliferation, and regeneration under metabolically active conditions.

Recent emerging data show that molecular dysregulation of the Hippo pathway contributes to the pathogenesis of several metabolic diseases such as obesity, T2D, fatty liver, and cardiovascular disorders; harnessing Hippo signaling could be a promising therapeutic approach for the therapy of metabolic disease.

Obesity tips to a systematic failure in the overall metabolic regulation; it is a major global health problem and is linked to several metabolic diseases such as **type 2 diabetes** (T2D) (see Glossary), **nonalcoholic fatty liver disease** (NAFLD), and cardiovascular disorders. The prevalence of such metabolic disorders has increased dramatically worldwide [1–5]. For instance, with an estimated number of over 420 million affected people and dramatic increases all over the world, diabetes has already gained epidemic status [6]. Although they share common molecular pathogenic signatures such as chronic low-grade inflammation [7], the underlying signaling pathways and molecular mechanisms are incompletely understood. Metabolism is a highly coordinated cellular function that can be flexibly regulated by signaling pathways to meet cellular requirements. Given the varied and enigmatic nature of the metabolic regulatory pathways, and the cellular and molecular causes of their dysregulation, the identification of signaling pathways that regulate the activity or expression of specific metabolic genes or pathways in general, together with an in depth-knowledge of their mechanisms of action underlying disrupted metabolic homeostasis in metabolic diseases, is of paramount importance and is urgently needed both for better understanding of disease pathogenesis and for the development of effective therapeutic interventions.

First discovered using genetic screens in *Drosophila* and subsequently established in mammals, the Hippo pathway has emerged as a key signal that controls organ size and tissue homeostasis by regulating cellular proliferation, survival, and regeneration [8]. Of particular interest is the emerging role of the Hippo pathway in the regulation of metabolic homeostasis at both the cellular and systematic levels. In addition to the pancreas and **pancreatic β cells** [9–17], multiple lines of evidence have uncovered the importance of Hippo signaling components such as MST, LATS, and YAP in cellular glucose and lipid metabolism, as well as in stress and metabolic adaptations in metabolically active organs including liver [18–23], fat [24–29], and heart [30–34] both under physiological conditions and in metabolic disorders. In the present review we discuss recent

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Declaration on the contribution of the candidate to a multi-author articles which are included in Chapter 2 in the submitted doctoral thesis

Publication I: Neratinib protects pancreatic β-cells in diabetes

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 25%
Experimental work and/or acquisition of (experimental) data:	ca. 25%
Data analysis and interpretation:	ca. 25%
Preparation of Figures and Tables:	ca. 25%
Drafting of the manuscript:	ca. 10%

Publication II: Inhibition of PHLPP1/2 phosphatases rescues pancreatic β -cells in diabetes

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 30%
Experimental work and/or acquisition of (experimental) data:	ca. 40%
Data analysis and interpretation:	ca. 40%
Preparation of Figures and Tables:	ca. 35%
Drafting of the manuscript:	ca. 10%

Publication III: PHLPP1 deletion restores pancreatic β-cell survival and normoglycemia in the db/db mouse model of obesity-associated diabetes

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 40%
Experimental work and/or acquisition of (experimental) data:	ca. 50%
Data analysis and interpretation:	ca. 70%
Preparation of Figures and Tables:	ca. 60%
Drafting of the manuscript:	ca. 80%

Appendix: Publication 1: The Hippo kinase LATS2 impairs pancreatic β-cell survival in diabetes through the mTORC1- autophagy axis

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 25%
Experimental work and/or acquisition of (experimental) data:	ca. 25%
Data analysis and interpretation:	ca. 25%
Preparation of Figures and Tables:	ca. 25%
Drafting of the manuscript:	ca. 10%

Appendix: Publication 2: LDHA is enriched in human islet alpha cells and upregulated in type 2 diabetes

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 10%
Experimental work and/or acquisition of (experimental) data:	ca. 10%
Data analysis and interpretation:	ca. 10%
Preparation of Figures and Tables:	ca. 10%
Drafting of the manuscript:	ca. 10%

Appendix: Publication 3: mTORC1 signaling: a double-edged sword in diabetic β-cells

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	/
Experimental work and/or acquisition of (experimental) data:	/
Data analysis and interpretation:	/
Preparation of Figures and Tables:	ca. 80%
Drafting of the manuscript:	ca. 20%

Appendix: Publication 4: mTORC2 signaling: a path for pancreatic β-cell's growth and function

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	/
Experimental work and/or acquisition of (experimental) data:	/
Data analysis and interpretation:	/
Preparation of Figures and Tables:	ca. 80%
Drafting of the manuscript:	ca. 20%

Appendix: Publication 5: Hippo signaling: key emerging pathway in cellular and whole-body metabolism

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	/
Experimental work and/or acquisition of (experimental) data:	/
Data analysis and interpretation:	/
Preparation of Figures and Tables:	ca. 80%
Drafting of the manuscript:	ca. 30%

Date:

Signature:

Acknowledgement

Early in my scientific career I realized that a person's achievements reach beyond a groundbreaking idea. Fundamental is the supporting network of people, who advise, complement, and accompany one another. Inside and outside of academia.

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