The Ubiquitin-Proteasome System (UPS) affects β-cell survival and function

Dissertation

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Abstract

Loss of insulin-producing pancreatic β-cells is the hallmark of both type 1 diabetes and type 2 diabetes. The mechanism and the components involved in β-cell death and failure are not yet fully clarified. Identification of key signaling components that promote β-cell death, understanding their mechanisms of action in detail is crucial in disease pathogenesis as well as for novel therapeutic interventions to halt β-cell failure during development and progression of diabetes. The ubiquitin-proteasome system (UPS) regulates the stability of many proteins involved in important cellular processes: cell cycle progression, cell differentiation, cell signaling pathways and apoptosis. In this work, I identified two genes within the proteasomal protein control system that are dysregulated in β-cells under diabetic conditions; F-box protein 28, a substrate recruiting a component of the Skp1-Cul1-F-box (SCF) ligase complex (SCF^{FBXO28}) and the deubiquitinase USP1. Both UPS components have an important function in β-cell survival in diabetes.

F-box only protein 28 (FBXO28) is part of the ubiquitination machinery, namely of the E-3 Ubiquitin Ligase complex that recruits proteins for degradation or for altering their localization or functional activities. My results show that FBXO28 protein levels were reduced under diabetic conditions. Loss of FBXO28 induced β-cell death, whereas its overexpression improved β-cell survival, and regulated expression of β-cell transcription factor NEUROD1 without altering insulin secretion as well as of several β-cell identity and functional genes. This suggests FBXO28 acts as a pro-survival protein in β-cells.

On the contrary, Ubiquitin-specific protease 1 (USP1), a member of the USP family and a well-known deubiquitinating enzyme (DUB) impairs β-cell survival in diabetes. USP1 is responsible for removing ubiquitin from substrate proteins and thus influences cellular processes such as survival, differentiation, immunity, and DNA damage response (DDR).

Genetic depletion or pharmacological inhibition of USP1 blocked β-cell death in several experimental models of diabetes *in vitro* and *ex vivo*. While DDR signals were elevated in

diabetes, USP1 inhibition attenuated the DDR in islets suggesting that the anti-apoptotic action of USP1 inhibition is mediated through suppression of DDR. I have identified a novel function of USP1 in the control of β-cell survival as potential therapeutic target for the suppression of β-cell death in diabetes.

Taken together, my data highlight the importance of an appropriate expression and activation of ubiquitin-proteasome components for pancreatic β-cell survival. My results prove that the ubiquitin-proteasome plays a key role in β-cell survival/failure in diabetes. Further in-depth understanding of the UPS system in β-cells and establishing its pathways would open up novel approaches towards diabetes therapy.

Zusammenfassung

Der Verlust von insulinproduzierenden pankreatischen β-Zellen kennzeichnet sowohl Typ-1 als auch Typ-2 Diabetes. Der Mechanismus sowie die beteiligten Komponenten in der β-Zelle bei Tod und Versagen sind jedoch noch immer nicht vollständig geklärt. Somit ist die Identifizierung wichtiger Signalkomponenten, die zum β-Zelltod führen, entscheidend für das Verständnis der Pathogenese von Krankheiten sowie für neue therapeutische Interventionen, um das Versagen von β-Zellen während der Entwicklung von Diabetes aufzuhalten.

Das Ubiquitin-Proteasom-System (UPS) reguliert die Stabilität von Proteinen, die an wichtigen zellulären Prozessen beteiligt sind: Zellzyklusprogression, Zelldifferenzierung, Zellsignalwege und Apoptose.

In dieser Arbeit identifizierte ich zwei Gene innerhalb des proteasomalen Proteinkontrollsystems, die in β-Zellen unter diabetischen Bedingungen fehlreguliert sind; FBXO28 (SCF^{FBXO28}), ein Substrat für den Skp1-Cul1-F-box (SCF) ligase complex (SCF^{FBXO28}), und die De-Ubiquitinase USP1. Beide UPS-Komponenten haben eine wichtige Funktion in der Regulation von β-Zellüberleben unter pathophysiologischen Bedingungen im Diabetes.

Das F-Box-only-Protein 28 (FBXO28) ist Teil der Ubiquitinylierungs-Maschinerie, nämlich des E-3-Ubiquitin-Ligase-Komplexes, der Proteine für den Abbau rekrutiert sowie deren zelluläre Lokalisation und Funktionalität beeinflusst.

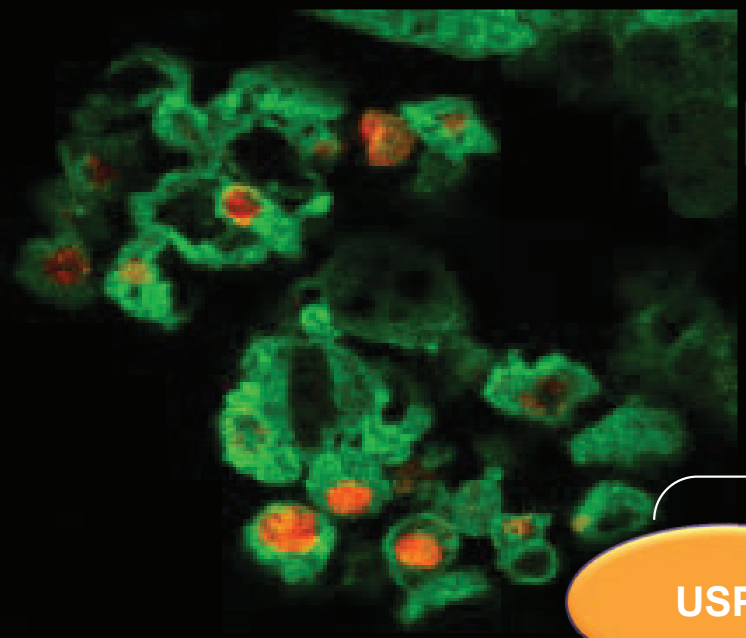
Meine Ergebnisse zeigen, dass die FBXO28-Proteinspiegel unter diabetischen Bedingungen erniedrigt sind. Der Verlust von FBXO28 potenziert den β-Zelltod, während dessen Überexpression das β-Zell-Überleben verbessert und die Expression des β-Zell-Transkriptionsfaktors NEUROD1 steigert, ohne jedoch die Insulinsekretion sowie mehrere β-Zellidentitäts- und -funktionsgene zu beeinträchtigen. Dies deutet auf die Funktion von FBXO28 als pro-Überlebens-Protein in β-Zellen hin.

Im Gegensatz dazu fördert die Ubiquitin-spezifische Protease 1 (USP1), ein Mitglied der USP-Familie und bekanntes Deubiquitinierungsenzym (DUB), das β-Zellversagen bei Diabetes. USP1 ist verantwortlich für die Entfernung von Ubiquitin von Substratproteinen und beeinflusst damit zelluläre Prozesse wie Überleben, Differenzierung, Immunität und DNA-Reparatur.

Genetische oder pharmakologische Inhibierung von USP1 blockierte β-Zelltod in mehreren experimentellen Modellen von Diabetes in vitro und ex vivo. Während DNA-Reparatur-Signale bei Diabetes erhöht waren, dämpfte die USP1-Inhibierung die DNA-Reparatur in Inseln. Dadurch kann man davon ausgehen, dass die überlebensfördernde Wirkung der USP1 inhibition mittels DNA-Reparatur-Reduktion ausgelöst wird. Mit meinen Ergebnissen habe ich eine neue Funktion von USP1 bei der Kontrolle des β-Zell-Überlebens als potentielle Therapie zur Unterdrückung des β-Zelltods bei Diabetes identifiziert.

Zusammenfassend unterstreichen meine Daten die Bedeutung einer kontrollierten physiologischen Expression und Aktivierung von Ubiquitin-Proteasom-Komponenten für das Überleben pankreatischer β-Zellen. Meine Ergebnisse belegen, dass das Ubiquitin-Proteasom eine Schlüsselrolle beim Überleben und beim Versagen von β-Zellen im Diabetes spielt. Ein tieferes Verständnis des UPS-Systems in β-Zellen und die Etablierung neuer unter- und übergeordneter Signalwege würden neue Ansätze für die Diabetes-Therapie eröffnen.

Targeting the Ubiquitin-Proteasome System in the pancreatic β-cell



Diabetes

Pancreatic β-cell death

USP1

FBXO28

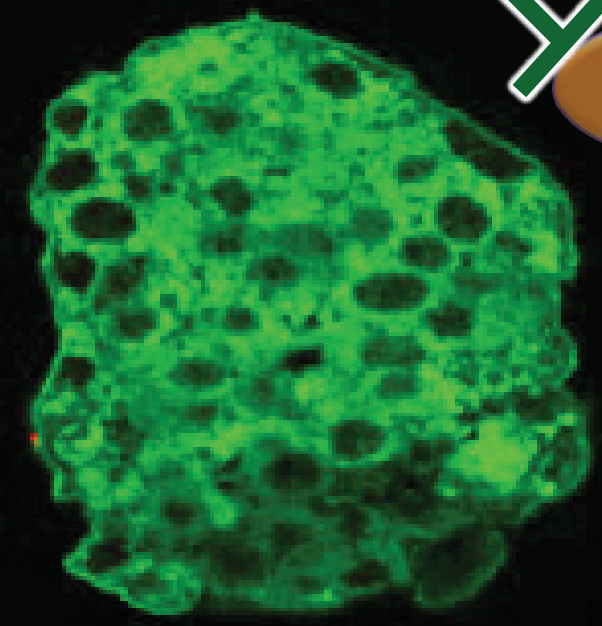
A pro-diabetic milieu

USP1 inhibition

FBXO28 over expression

USP1

FBXO28



β cell survival

Contents

Abbreviations

CDK1/2	Cyclin-dependent kinase ½
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GSIS	Glucose-stimulated insulin secretion
hIAPP	Human islet amyloid polypeptide
IAPP	Islet amyloid polypeptide
IFN-γ	Interferon gamma
IL-1β	Interleukin 1 beta
MOI	Multiplicity of Infection
NDRG1	N-Myc downstream regulated 1
NeuroD1	Neurogenic differentiation 1
NF-κB	Nuclear factor kappa B
PARP	Poly (ADP-ribose) polymerase
ROS	Reactive oxygen species
SCF	Skp1-Cul1-F-box
SKP1	S-Phase Kinase associated protein 1
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
TCA	Tricarboxylic acid
TNFα	Tumor necrosis factor alpha
Ub	Ubiquitin
UPS	ubiquitin-proteasome system

1. Introduction

1.1. Diabetes Mellitus

Diabetes mellitus (DM) is perhaps one of the oldest diseases known to humans; it had been first mentioned in Egyptian manuscript almost 3000 years ago. DM is a metabolic disease ensuing from the defects in insulin secretion, insulin action or a combination of both. Insufficient insulin thus ends up in chronic hyperglycemia with unsettling influences of carbohydrate, fat and protein metabolisms. It is reported that by 2030, 552 million individuals will have DM [1]. DM is categorized into four types based on the etiology and clinical features. They are **1**) Type 1 diabetes mellitus (T1DM) **2**) Type 2 diabetes mellitus (T2DM), **3)** Other specific types of diabetes, and **4)** Gestational diabetes mellitus (GDM) [2].

1.1.1. Type 1 diabetes mellitus (T1D)

T1D also referred as immune-mediated diabetes or autoimmune diabetes, represents 5-10% of the population with diabetes [2]. T1D is a chronic autoimmune disease and is described by absolute insulin deficiency due to pancreatic β-cell loss and thereby resulting in hyperglycemia [3]. Even though the age of symptomatic onset is generally at some stage in early life or adolescence, symptoms can at times grow considerably later. In spite of the fact that the etiology of T1D is not totally comprehended, the pathogenesis of the disease includes T cell-intervened destruction of β-cells [4].

We solely understand that autoimmunity is the dominating effector system of T1D. T1D develops in genetically vulnerable people, because of an environmental trigger. Current genetic information points towards several susceptibility genes, which all are involved somehow in the interaction of T-cells with β-cells: HLA, insulin, PTPN22, IL2Ra, and CTLA4 [3]. Epidemiological, clinical and basic studies have found a triggering role for enteroviruses. T1D prevention would require recognition of the earliest events within the process. Until now, autoantibodies are most generally utilized as serum biomarker; however T-cell readouts and metabolome research would possibly enhance and convey a forward diagnosis [3]. Islet-targeting autoantibodies; 65 kDa glutamic acid decarboxylase, insulinoma-associated protein 2, and zinc transporter 8 are proteins related to secretory granules in β-cells and are biomarkers of T1D autoimmunity

expressed years before T1D onset and are utilized to recognize patients at risk of developing T1D. As described in figure 1, the pathogenesis of T1D proceeds in four different stages [5]. A curative treatment isn't available, and patients rely upon lifelong insulin injections; novel strategies of insulin therapy, which include insulin pumps, continuous glucose monitoring, and hybrid closed-loop systems have been developed. Even though extensive glycemic control has reduced the incidence of microvascular and macrovascular complication, most of the people with T1D are yet building up these microvascular and macrovascular complications [4].

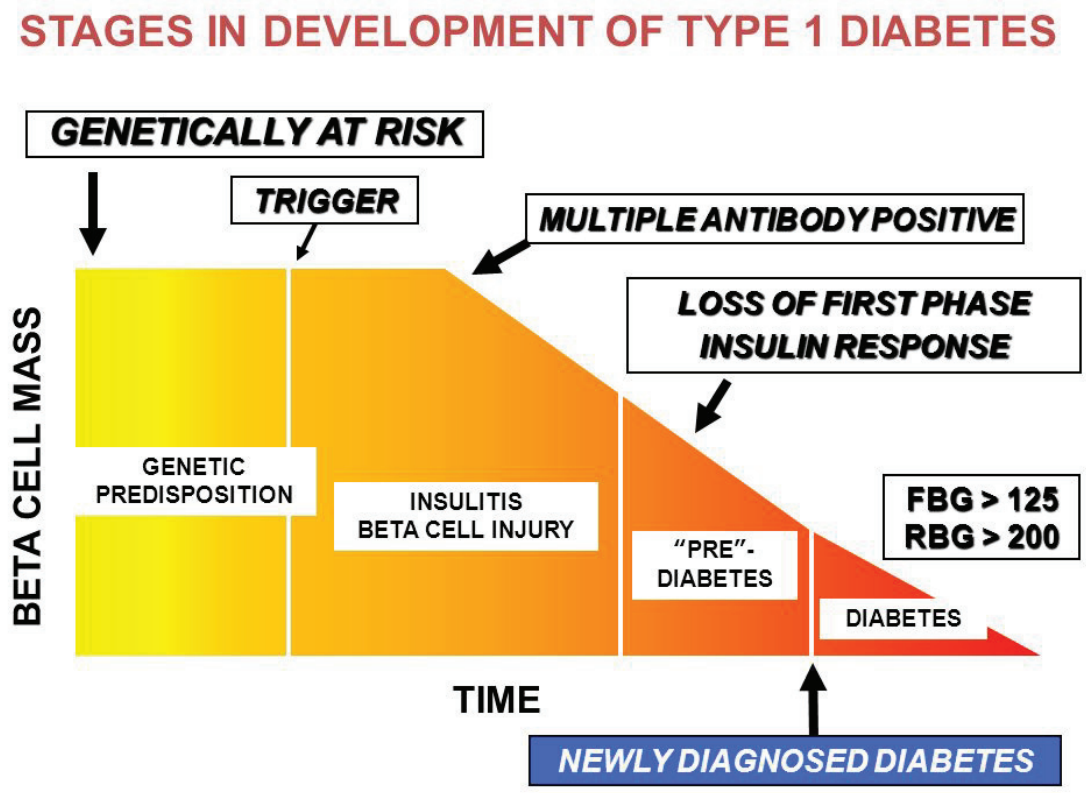


Figure 1. Stages in the development of type 1 diabetes *(taken from[5]). Theoretically, the development of T1D can be divided into six stages, starts with genetic susceptibility and finally ends with total β-cell destruction. Stage 1 is genetic susceptibility, stage 2 environmental factors that can trigger the development of β-cell immunity. In stage 3 the autoimmune phenomena appear, autoantibodies produced against β-cells results in β-cell damage. Stage 4 represents a loss of first phase insulin secretion which leads to the development of hyperglycemia (higher blood glucose levels) a pre-diabetes stage 5. By the time that over glycemia and extensive β-cell destruction has already presented in stage 6 most of islets have no β-cells.*

Predominant studies are required to accomplish early diagnosis, prevent β-cell loss and develop better treatment alternatives. Present preventive clinical trials principally concentrate on inhibition of autoimmunity and environmental triggers. Therapeutic trials examine the effectuality of antigen-specific and antigen-nonspecific immune interventions, however also encompass restoration of the affected β-cell mass by islet transplantation, regeneration, and neogenesis [3, 4].

1.1.2. Type 2 diabetes mellitus (T2D)

T2D is the most typical form of diabetes and accounts for 90-95% of total diabetes mellitus cases. T2D is characterized by a β-cell failure (loss of mass and/or function), usually together with insulin resistance. Prior to the prognosis of T2D, people can stay undiagnosed for a long time due to the progressively developing hyperglycemia. This increases the chance of complications because of the prolonged exposure to elevated glucose levels in the circulation. At an early phase of the disease, the circulating insulin levels are high to compensate for the diminished insulin sensitivity. As the disease advances, β-cells secrete inadequate levels of insulin resulting an imbalance in glucose homeostasis that ultimately leads to T2D [2]. Medical management of T2D includes initially and most importantly a change in lifestyle, such as modifications in diet, magnified physical activity [6], and, if the former is not successful, medical treatment that targets to improve the insulin secretion and insulin sensitivity in peripheral tissues [7, 8]. In advanced phases of T2D, the patients may require insulin to regulate their blood glucose levels.

The cause of T2D is not clearly understood; it is the result of a complicated interaction between genetic predisposition and environmental elements. Geneticists have up to date identified approximately 70 genomic regions related to T2D [9], each of them just clarifies a little part of the genetic background of T2D [10]. The greater parts of these identified genes appear to be involved in the regulation of pancreatic β-cell mass and function related to insulin secretion, which also demonstrates the significance of insulin-secreting β-cells in the progression of T2D [11]. Environmental factors conducive to the progression of T2D consist of excessive caloric consumption and a sedentary lifestyle resulting in weight problems, and as on account of T2D, the prevalence of obesity is likewise growing across the globe. Obesity is firmly connected with

the increased risk of developing insulin resistance that results in lack of insulin response in peripheral tissue [12]. T2D is a most common disease among adults however a stressing point is the recent fast increment of T2D in children and adolescents [13]. Changes in the circulating lipid profile have been seen in obese people and there is mounting proof that the increased levels of circulating free fatty acids in skeletal muscle lead to insulin resistance; in myocardium, it damages cardiac function; and in pancreatic islets, it causes β-cell dysfunction, apoptosis, and contributes to the development of β-cell failure and T2D [14].

1.2. Insulin

Insulin is a peptide hormone secreted by β-cells of the pancreatic islets of Langerhans and its secretion is stimulated by intracellular signals obtained from nutrient metabolism where glucose stimulus is predominant [15]. Insulin is vital for the maintenance of glucose homeostasis. Insulin regulates the carbohydrate, protein, and lipid metabolisms and promotes growth and cell division via its mitogenic properties [16-18].

1.2.1. Discovery of insulin

In 1889 German researchers Minkowski and von Mering discovered that total pancreatectomy of their experimental animals was responsible for the progression of severe diabetes. Their hypothesis was that the pancreatic secreted substance was in charge of metabolic regulation [16, 19]. Later, other researchers reinforced the hypothesis stating that diabetes is caused by the demolition of the islets of Langerhans [20]. The name “Insuline” was suggested by the Belgian researcher de Meyer in 1909, which was again confirmed by the British scientist Schaefer in 1916 [19].

Finally, after a decade in 1921, insulin was isolated, purified and obtained in a form capable of therapeutic usage. In May 1921, Toronto surgeon Banting and his student Best under the guidance of McLeod, Professor of carbohydrate metabolism, started their experiments in dogs. They injected chilled saline pancreatic extracts to diabetic dogs that underwent pancreatectomy and they observed lowered blood glucose levels [21]. In December 1921, Collip, a biochemist who joined the team, additionally showed that this extract reestablished hepatic glycogen mobilization and the ability to clear the ketones. In January 1922, the first human experiments

were started in a 14-year old teenager with diabetes. The clinical symptoms and biochemical abnormalities of diabetes were basically reversed in this child after injecting the pancreatic extract. In May 1922, this dynamic component was named insulin and the experimental results were presented to the Association of American Physicians [22]. In early 1923, Eli Lilly started the production of porcine insulin purified through iso-electric precipitation in commercial quantities. In 1923, Banting and McLeod received the Nobel prize [16, 23].

1.2.2. Insulin synthesis and release

In humans, the insulin gene is encoded on chromosome 11 [24]. It is present in β-cells of the pancreatic islets as a proinsulin precursor. Insulin synthesis begins with the transcription of the insulin gene followed by the translation of insulin mRNA into precursor pre-proinsulin in the ribosomes of rough endoplasmic reticulum. Pre-proinsulin is cleaved into proinsulin in the endoplasmic reticulum and transferred to Golgi apparatus through the secretory vesicles. With the help of aqueous zinc and the calcium-rich environment of the Golgi apparatus, soluble zinc-containing proinsulin hexamers are formed [25]. Proinsulin is converted into insulin and c-peptide outside the Golgi by prohormone-converting enzymes PC1/3 and PC2. The two zinc containing hexamers of mature Insulin and the detached c-peptide is stored in the secretory vesicles and are released upon beta cell stimulation [16]. Insulin secretion is biphasic in response to glucose stimulus; the initial swift phase of insulin secretion followed by a less intense but sustained release [26].

1.2.2.1. Factors Regulating Insulin secretion

The major stimulator of insulin biosynthesis and secretion is glucose. Nevertheless, different factors, for example, amino acids, fatty acids, glucagon-like peptide-1 (GLP-1), acetylcholine, pituitary adenylate cyclase-activating polypeptide (PACAP), glucose-dependent insulinotropic polypeptide (GIP), and numerous other agonists collectively influence the synthesis and secretion processes of insulin [26-29].

1.2.2.2. Mechanism of insulin secretion

Glucose is the main regulator of insulin secretion, high glucose levels induce the first-phase of glucose-stimulated insulin secretion from β-cell secretory granules. The high blood glucose levels trigger the pathway with the specific glucose transporters GLUT-1 in human and GLUT-2 in rodents in the β-cell [30, 31]. As described in figure 2, the entry of glucose into the β-cell is sensed by glucokinase, which quickly phosphorylates glucose and further metabolizes it to pyruvate through glycolysis involving a series of enzymes. Pyruvate is further processed in the mitochondria via tricarboxylic acid cycle (TCA cycle), to produce ATP. The high ATP/ADP ratio results in the closure of the ATP-sensitive potassium (KATP) channels leads to membrane depolarization and activation of voltage-dependent Ca^{2+} channels. Ca^{2+} influx activates the fusion of a readily releasable pool (RRP) of insulin-containing vesicles, leading to the quick release of insulin [32, 33]. This mechanism is particularly accounted for the first phase of insulin secretory response; it begins within minutes of glucose stimulation and lasts for 5-10 mins [34]. At the point when the RRP of insulin-containing vesicles are exhausted, these are refilled from a reserve pool (RP) [33]. The augmentation of this insulin secretory response occurs by both KATP channel-independent Ca^{2+}-dependent pathway and KATP channel-independent Ca^{2+}-independent pathways of glucose action. Other mediators that influence insulin release are activation of phospholipases and protein kinase C (e.g. by acetylcholine), induction of adenylyl cyclase function and triggering of β-cell protein kinase A. This mechanism might be enacted by hormones, along with vasoactive intestinal peptide (VIP), GLP-1, PACAP, and GIP. These components seem to play a crucial role in the second phase of glucose-stimulated insulin secretion [26], which is more sustained and is slowly expanded over an hour [33]. In healthy individuals, the balance of insulin secretion from β-cells and insulin action in peripheral tissue is maintained to control the blood glucose levels. In T2D, multiple steps of glucose-stimulated insulin secretion are impaired and loss of the first phase of insulin secretion is the main pathogenic event [35]. A lower level of glucokinase expression and a reduced insulin secretion were reported in islets from patients with T2D [36].

1.2.3. Insulin signaling

The principal function of the secreted insulin from β-cells is to stimulate glucose uptake in peripheral tissues, inhibit glucose synthesis inside the liver, inhibit fat breakdown and promote fat synthesis in adipose tissue. Insulin acts upon binding to the transmembrane insulin receptor (IR) on the target cell surface. The insulin receptor comprises two α-subunits and two β-subunits, which are connected by disulphide bonds. Insulin binding to the α-subunit results in autophosphorylation of the β-subunit and activation of insulin receptor substrate (IRS) 1 and 2 which in turn activate the phosphatidylinositol-3-kinase (PI3K)-AKT pathway, resulting in translocation of glucose transporter 4 (GLUT-4) to the target cell surface [38]. GLUT-4 is in charge of intra-cellular transport of glucose from the blood into adipose tissue and muscle. As mentioned earlier, insulin action and glucose uptake inhibits fat catabolism but promotes the synthesis, whereas in skeletal muscle triggers the glycogen synthesis [16, 38]. Defects in insulin secretion, insulin action or combination of both results in hyperglycemia and eventually end up in development of the metabolic disorder diabetes.

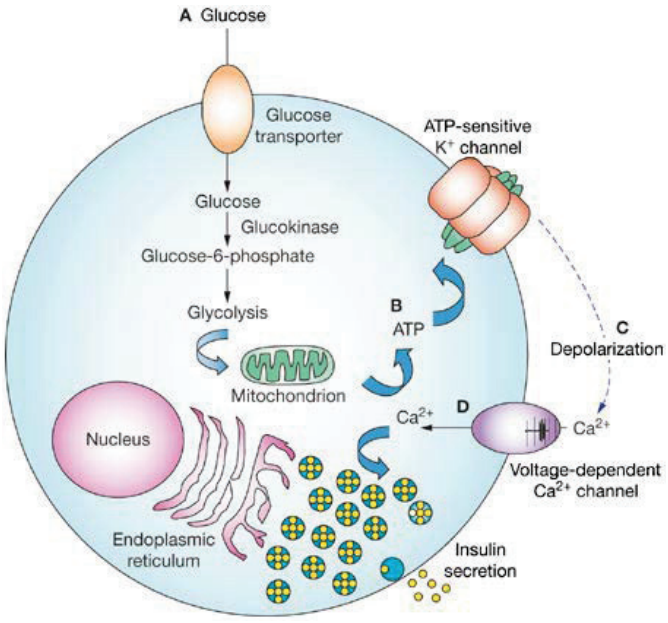


Figure 2. Glucose-stimulated insulin secretion in pancreatic β-cells *(Taken from [37]). (A) Glucose entry into the cell and its metabolism by glucokinase (B) increased intracellular ATP–ADP ratio results in closure of ATP-sensitive potassium channels, (C) cell membrane depolarization and the opening of voltage-dependent Ca^{2+} channels. (D) The resulting increase in cytosolic Ca^{2+} concentration triggers insulin release.*

1.2.4. Pancreatic β-cell death

Apoptosis or programmed cell death is a hallmark of β-cell failure in both autoimmune T1D and also in T2D [39, 40]. Post-mortem studies from pancreases report a 25-50% decrease in β-cell mass in T2D patients [40].

Apoptosis is a well-controlled procedure; it is crucial for maintaining cell homeostasis throughout development but at the same time, it is a defense system to expel damaged cells [41]. Apoptotic death is induced by means of an extrinsic, receptor-mediated signaling pathway or an intrinsic, mitochondrial-mediated signaling pathway. The intrinsic apoptotic pathway occurs in response to numerous injuries, as shown in figure 3, together with DNA-damage, mitochondria dysfunction because of ROS or Ca2+ overload and endoplasmic reticulum stress (ER stress). The caspases belong to the protease family, are master cell death executors and a key regulator of apoptosis [42]. Apoptosis is most likely the main form of β-cell death in both T1D and T2D. Cytokine and nutrient-induced β-cell death mechanisms are reported to be involved in both forms of diabetes. T1 and T2 diabetes share the final common pathway activation involves in interleukin (IL)-1β, NF-kB, and Fas pathways [40]. In T1D, β-cells are the target of autoimmune assault [43]. The β-cell death in T1D, perhaps the result of direct interaction with activated macrophages and T-cells, or exposure to cytokines, free radicals, and nitric oxide (NO) secreted by these activated cells induce β-cell gene networks activation under the regulation of NF-kB and STAT-1. Activated NF-kB results in the production of chemokines, nitric oxide, and also deplete the endoplasmic reticulum calcium. The β-cell death results with activation of mitogen-activated protein kinases, through ER stress induction and mitochondrial death signals release [40]. Chronic exposure to high glucose, free fatty acids, islet amyloid polypeptide deposits and ER stress are known to responsible for β-cell dysfunction and also which may lead to β-cell death in T2D [44]. High glucose concentrations can lead to β-cell death and it could be a key mechanism leads to β-cell dysfunction in T2D. Saturated fatty acids, likely palmitate in β-cells and cell lines result in the induction of apoptosis and ER stress pathway activation. Free fatty acids with high glucose concentrations (glucolipotoxicity) can also induce β-cell death [44]. Islet amyloid polypeptide deposits also lead to β-cell death and ER stress in T2D [45]. ER stress plays a crucial in the mediation of β-cell apoptosis in response to high

glucose, free fatty acids, and IAPP [46]. Prolonged or persistent ER stress results in the generation and accumulation of ROS which can mediate β-cell apoptosis [47].

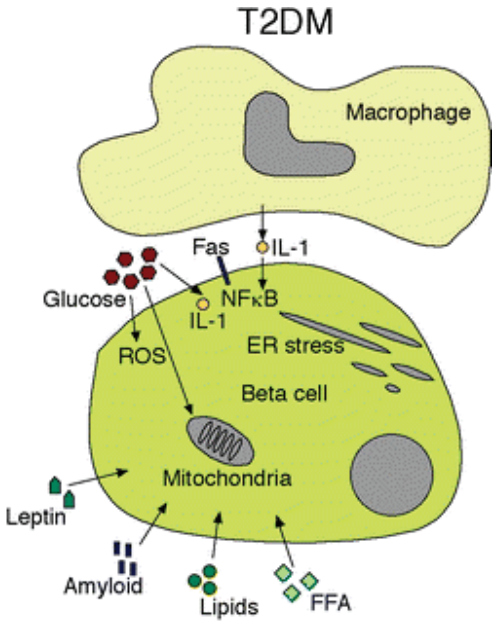


Figure 3. Pancreatic β-cell death in T2D *(Taken from[44]). The major reason for β-cell death is not yet known. Glucose, islet amyloid polypeptide saturated fatty acids, and IL1-β reported to be involved in β-cell apoptosis. These stimuli are predicted to induce ER stress and oxidative stress, which leads to β-cell apoptosis.*

1.2.5. Endoplasmic reticulum stress-induced β-cell death

β-cells secrete large quantities of insulin. Insulin mRNA translation in response to glucose stimulus constitutes 50% of the total protein synthesis [48, 49]. The endoplasmic reticulum is the site of protein synthesis, folding and maturation. The ER is equipped with a quality-control system that recognizes the misfolded proteins and targets them for degradation via the ubiquitin-proteasome system [50]. When excessive misfolded proteins are generated, the aggregation of the protein occurs and results in intracellular accumulation of protein aggregates. [51].

Effectual evacuation of misfolded proteins through the endoplasmic reticulum-associated degradation (ERAD) is important to preserve cells from ER stress. This is often done through different steps. As described in figure 4, initially, if a protein fails quality control, it is eliminated

from the ER via retrograde translocation. Secondly, several ubiquitin proteins covalently bound to the substrate or target protein. Third, the polyubiquitinated protein migrates to the 26S proteasome. Fourth, the ubiquitin chains are expelled from the misfolded target protein by a deubiquitinating DUB, and hence it's far ultimately rendered accessible for degradation by passing through the 26s proteasome[50]. Therefore, the ubiquitin-proteasome pathway is one of the systems for intracellular protein degradation, control and for the evacuation of protein aggregates in β-cells [51].

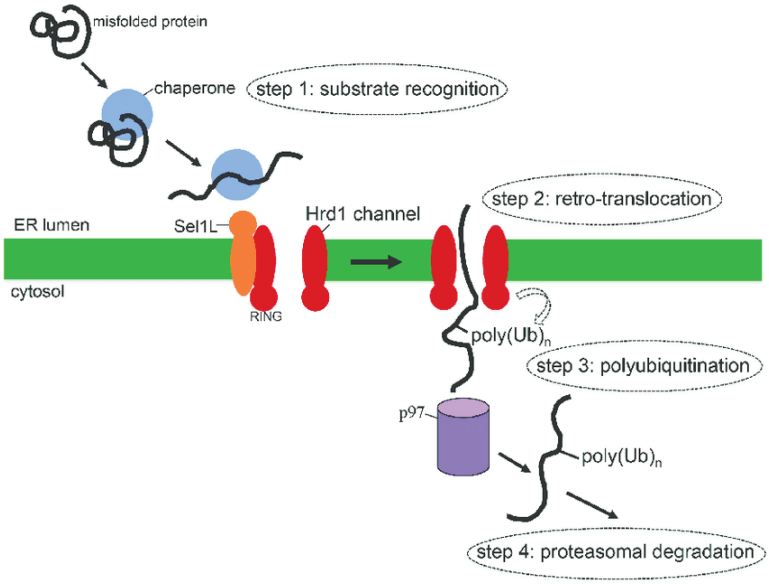


Figure 4. ERAD pathway *(taken from [52]). Step 1: ER chaperons recognize misfolded proteins, step 2: misfolded protein retro-translocation across the ER membrane by crossing the retrotranslocation channel (Sel1L-Hrd1 membrane complex), step 3: Hrd1 poly-ubiquitinates the misfolded protein in cytosol, step 3: in the final step the substrate is extracted by p97 into the cytosol followed by proteasomal degradation.*

1.3. The intracellular protein control and degradation system

Every single biochemical process of an individual cell requires molecules that self-assemble into functional structures and a proper quality control system that is vital to maintain the reactions in cellular environment [53]. Proteins, which control folding, trafficking, degradation, repair, and maintenance, are crucial for every biological pathway. The functions of proteins are as diverse as their structures. Maintaining the proteome integrity and protein homeostasis is essential for cell survival [54]. The newly synthesized proteins undergo several conformational modifications to attain a precise three-dimensional form, in order to be functionally active. [55]. Cells have a

well-established machinery to detect and preserve the healthy proteome [56]. Maintaining proteostasis or protein homeostasis deals with several other strategies that focus on refolding, degradation and sequestering misfolded proteins [57]. The proteostasis elucidates the causes of diseases related to protein misfolding and protein degradation that result in loss of functional phenotypes. The healthy proteome or homeostasis can be achieved by evaluating the proteins regularly, i.e., degrading the old, misfolded and unnecessary proteins [58]. Molecular chaperones recognize and promote refolding of misfolded proteins. If the refolding is not possible, chaperons promote the degradation of misfolded proteins via ubiquitin-proteasome system [59]. Intracellular protein degradation is a very complex process due to the unevenness and concentration of proteins within the cellular environment [60]. Most of the intracellular proteins are highly stable but others are unstable and swiftly degrade, in order to regulate their precise function [58]. Intracellular protein quality control regulates the cell by the immediate removal of damaged and misfolded proteins. This is very important to inhibit the formation of toxic aggregates and control the inheritance of damaged and or aggregation of species within the cell [61].

Intracellular protein degradation mechanism is very specific; degradation and folding together are defined as protein quality control. In eukaryotes, the selective protein degradation is carried out by the ubiquitin-proteasome system (UPS); which rapidly degrades the majority of proteins including misfolded proteins. This makes the proteasome highly responsible for protein homeostasis [62].

1.4. Discovery of the Ubiquitin-Proteasome System

Earlier, protein degradation was assumed to be carried out by the lysosomal pathway [63]. The cytosolic protein degradation requires ATP energy for protein degradation, as the peptide bond hydrolysis does not require metabolic energy [64]. After many years, a cell-free system made researchers to discover the non-lysosomal protein degradation [65]. Hershko and his colleagues fractionated and purified the reticulocytes to identify the components involved in ATP-dependent cytosolic protein degradation. Their results were supported by genetic and in vivo studies on protein degradation [64] and led to the discovery of a highly specific, strongly precise, and tightly controlled ATP dependent process known as the Ubiquitin pathway (Ubiquitin

proteasome system (UPS)). The ubiquitin-dependent non-lysosomal protein degradation led to a new era of understanding protein degradation. In 2004, Ciechanover, Hershko, and Rose were awarded the Nobel Prize for their discovery of UPS [58, 66].

1.5. The Ubiquitination pathway

Ubiquitination as a post-translational modification regulates almost all biological pathways in eukaryotic cells including cell cycle, DNA repair, signal transduction, transcription, immune response, protein localization, protein quality control, and -especially- protein degradation [67]. Protein ubiquitination can be induced by several other upstream signals, for example, cell surface receptors are ubiquitinated by the stimulation of extracellular ligands [68]. For many nuclear and cytosolic proteins, ubiquitination is introduced by post-translational modifications such as phosphorylation but it is not universally required [69]. In the process of ubiquitination, proteins destined for degradation or ubiquitination are called as substrates and are covalently modified by attaching to ubiquitin molecule. Cellular proteins tagged with polyubiquitination on lys-48 and lys-29 are main targets for the 26S proteasome for degradation. Whereas, ubiquitin-tagged lys-63 carries the signal for DNA repair pathways, transcription, etc. [70]. Monoubiquitination results in signaling of several other biological processes like endocytosis and histone regulation etc. [71]. As figure 5 shows, ubiquitination is a signaling cascade that is carried out by a series of enzymes. Three classes of enzymes are involved in this process, E-1 ubiquitin activating enzyme activates the ubiquitin by utilizing ATP energy, then the activated ubiquitin is transferred on to E-2 ubiquitin-conjugating enzyme, and finally, E-3 ubiquitin ligase binds to enzyme E-2 and the substrate, and transfers the ubiquitin onto its target protein [67]. The protein ubiquitination is a reversible process; the deconjugation of ubiquitin from substrates is carried out by deubiquitinases (DUBs) [72].

Ubiquitination is a more complex process related to any other post-translational modifications. Ubiquitin polypeptide contains seven surface lysine residues (K6, K11, K27, K29, K33, K48, and K63) which all can be ubiquitinated and form a diversity of ubiquitin chain types [74]. The ubiquitin chains can be categorized into two types monoubiquitination and polyubiquitination as described in figure 6.

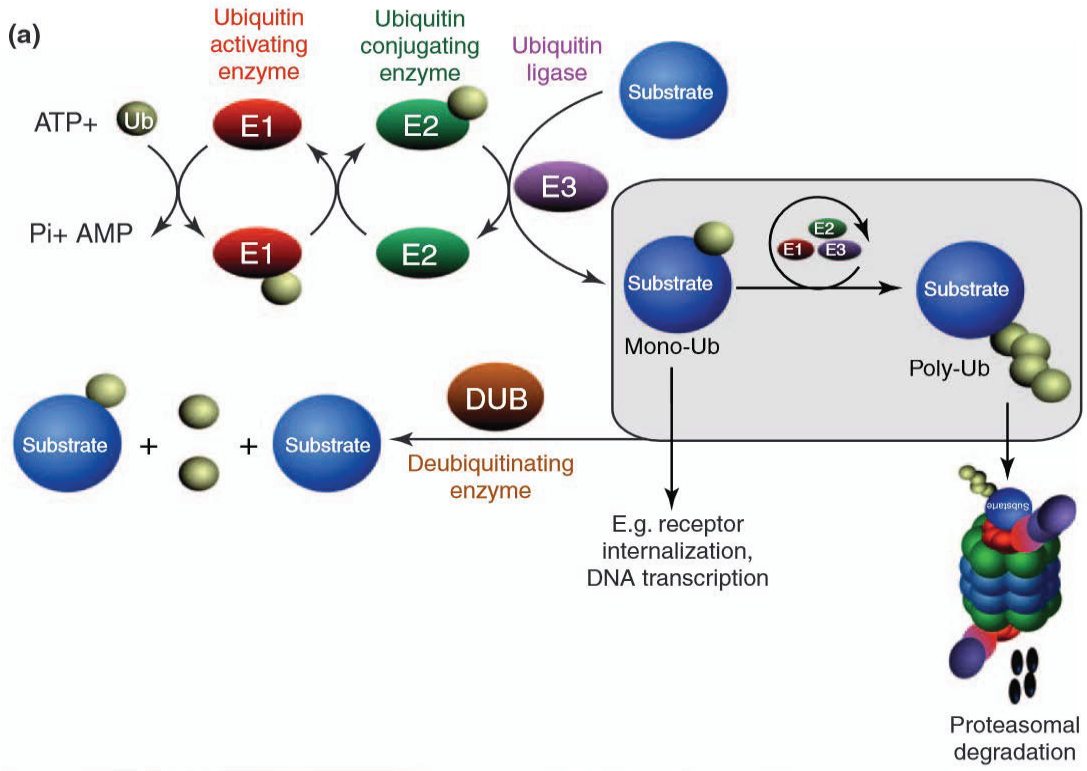


Figure 5. Ubiquitination pathway *(taken from[73]). During the first step of ubiquitin conjugation, ATP is used and E1 forms a thiol-ester intermediate with the terminal glycine of ubiquitin. Next, ubiquitin is transferred to E2 and finally, in the presence of an E3, ubiquitin is transferred to the target protein. Proteins are either mono-ubiquitinated or poly-ubiquitinated. Mono-ubiquitination of a protein usually leads to its internalization and DNA transcription. Polyubiquitination of a protein normally leads to proteasomal degradation. Protein ubiquitination is reversed by DUBs, which can completely deubiquitinate a protein or edit the extent of ubiquitination.*

1.5.1. Monoubiquitination

Monoubiquitination refers to the attachment of a single ubiquitin monomer to the substrate protein. Likewise, the addition of ubiquitin monomers to several lysine molecules of a substrate protein results in its multi-ubiquitination or mono-multi-ubiquitination. Monoubiquitination of proteins can result in protein internalization, trafficking, subcellular localization, lysosomal degradation and histone function [75-78], but cannot regulate protein degradation or turn over. A familiar mono-ubiquitinated protein is the phosphorylation substrate for epidermal growth factor receptor (EGFR) which is epidermal growth factor (EGF) receptor pathway substrate clone 15 (Eps15) [79]. Moreover, ubiquitin chain elongation on proteins needs monoubiquitination as a start to form polyubiquitinated proteins [80].

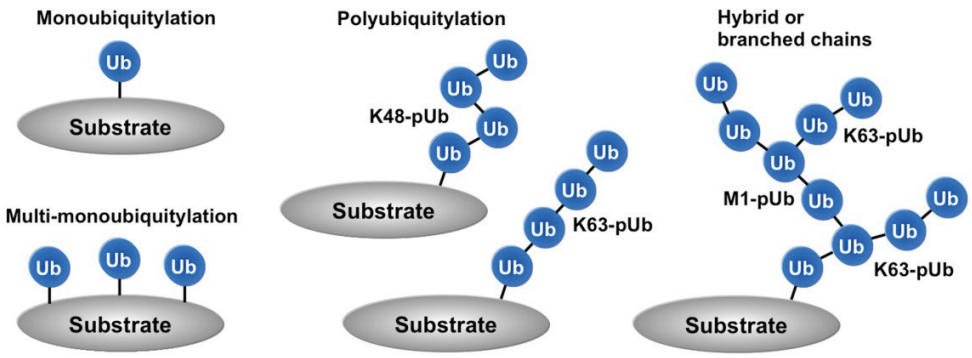


Figure 6. Different types of ubiquitylation *(taken from [81]). Monoubiquitylation, multi-monoubiquitylation, and polyubiquitylation. Polyubiquitylation is further subdivided into homotypic ubiquitylation (each ubiquitin chain containing just one type of ubiquitin linkage) or heterotypic ubiquitylation (comprising more than one type of ubiquitin chain). The latter is generally called as a hybrid, branched or mixed ubiquitin chains.*

1.5.2. Polyubiquitination

Addition of several ubiquitin monomers on to an amino acid of a protein results in a poly-ubiquitin chain formation. In poly-ubiquitination, several ubiquitins are linked through a specific lysine residue of ubiquitin. Unlike mono-ubiquitination, polyubiquitination allots several properties to the protein [82]. Ubiquitin polypeptide contains seven lysine residues and each lysine generates a specific poly-ubiquitin chain; well-studied poly ubiquitination types are Lys-48 and Lys-63 ubiquitin chains [82].

It is well known that Lys-48 linked polyubiquitin chains are involved in regulating protein degradation. It is widely accepted that proteasome, that recognizes and degrades the target protein contains Lys-48 linked chain composed of tetramers or oligomers of ubiquitin molecules [83, 84]. Thus the ubiquitin-proteasome pathway serves as a crucial mechanism for cellular protein turnover in eukaryotes [85]. This process results in protein degradation and the recycling of ubiquitin to cellular pools as monomeric ubiquitin moieties are achieved by proteasome-associated enzymes like deubiquitinases (DUBs) [86].

Several researchers stated that Lys-63 linked ubiquitin chains are non-degradative signals and Lys-63 polyubiquitinated proteins regulate several cellular functions including DNA damage repair, kinase signaling and activation, intracellular signaling and protein synthesis [87-89]. For

example, Lys-63 associated polyubiquitination of the transcription factor Myc results in activation of its multiple downstream target genes for tumor development [90]. Upon DNA damage, the Lys-63 poly ubiquitinated histones recruit proteins for DNA damage repair [91]. The Lys-63 linked polyubiquitin chain's role in recruiting proteins for proteasomal degradation is not yet fully discovered. Recent research also reported the accumulation of Lys-48 and Lys-63 polyubiquitinated proteins when the proteasome is inhibited and linked Lys-63 ubiquitination to protein degradation [92].

Lys11 and lys29 linked ubiquitin chains are generally considered as a secondary degradation signal, the existence is less in comparison to Lys-48 linked chains. Lys-11 linked ubiquitination regulates the degradation of cell cycle regulators during mitosis [93]. The Lys11 and lys-29 associated protein degradation is not only through proteasome, but also through lysosomal degradation and the ERAD (endoplasmic reticulum-mediated) pathway [94-96].

1.6. The Ubiquitin-proteasome machinery

1.6.1. The 26S proteasome

The 26S proteasome is a well-studied multi-catalytic complex belonging to the ATP dependent chambered proteases family. The proteasome is a very large structure with a molecular weight of about 2.5 MDa [84, 97] and acts as a degrading arm for ubiquitination process, hence crucial for regulated protein degradation of nuclear, cytosolic, and membrane proteins in all eukaryotes [98]. The proteasome-mediated degradation regulates the shelf-life of several cellular proteins that are involved in the important cellular process. Many regulatory proteins are short-lived proteins, proteasome pathway known to turn-over of several eukaryotic short-lived proteins. The well-known proteasome substrates are transcriptional regulators (IκB), c-Jun, P53 and cell cycle regulators (mitotic, G1, and S-phase cyclins) [85]. Their self-regulation is very crucial for protecting cellular homeostasis [99, 100]. The proteasome complexes of eukaryote cells are determined (both in the cell nucleus and cytoplasm) by specific particles in the endoplasmic reticulum and the cytoskeleton. Since the proteasome is the major cellular protease and is central to proteostatic mechanisms in the living cell, the structure and function of this regulated

complex are crucial for cellular processes. Whereas, its misregulation results in disease states [97].

1.6.2. Structure and function of the 26S proteasome

The 26S proteasome has two unique sub-complexes; the 20S core particle (CP) and the 19S regulatory particle (RP) as showed in figure 7. The 19S RP is a multifunctional sub-unit of the proteasome, which identifies, binds, deubiquitinates, unfolds, and translocates target proteins into an enclosed cavity formed by the CP [99]. In the CP, the target protein degrades into small peptides by the action of catalytic sites. Further, the peptidases act on the small peptides, degrade them into amino acids to be reused by the cell. The CP is barrel-shaped and is constructed by four axillary arranged seven sub-unit rings. The CP complex is a four subunit ring arrangement where two α-rings outside and two β-rings inside are designated as α1–7, β1–7, β1–7, and α1–7 [100, 101].

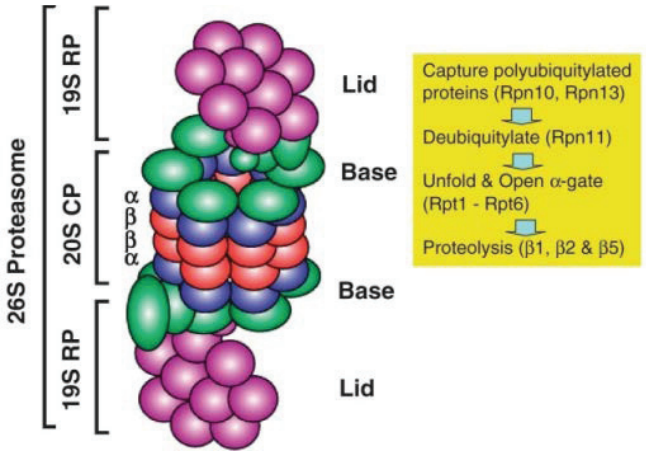


Figure 7. The 26S proteasome structure *(taken from [102]). CP, core particle (20S proteasome); RP, a 19S regulatory particle consisting of the base and lid sub-complexes;*

The outside α-rings of the CP form a pore-like structure, composed of seven similar but diverse subunits (α1–7) and serve as closely controlled gate for the entry of targeted proteins and for the removal of degraded products from the complex [103]. The inside two β-rings contain seven diverse β-subunits (β1–7); three of them β1, β2, and β7 with active sites with a series of

proteolytic specificities that include peptidyl-glutamyl-hydrolyzing or caspase-like activity, trypsin-like activity, and chymotrypsin-like activity [104, 105]. During synthesis, the catalytic β-subunits synthesize structures like precursors containing propeptides at the N-terminal. But during proteasome maturation, exposure of N-terminal catalytic threonine necessitates the removal of propeptides [105, 106]. The matured mammalian proteasome contains six proteolytic sites with three different proteolytic functions [99].

The 19S regulatory particle is further categorized into two subcomplexes; the base and the lid. The base contains six regulatory particles and ATPase subunits named Rpt1, Rpt2, Rpt3, Rpt4, Rpt5, and Rpt6, four regulatory particles and non-ATP sub-units named Rpn1, Rpn2, Rpn10 and Rpn13 also known as Adrm1 [107]. The six ATPase sub-units structured into a ring form and the non-ATP Rpn1, Rpn10, and Rp13 function as ubiquitin receptors that recognize the target proteins for the proteasome. The lid contains nine various Rpn sub-units; Rpn3, Rpn5-9, Rpn11, Rpn12, and Rpn13 also known as Dss1/Sem1, which generates a horse-shoe shaped structure. The lid serves as a deubiquitination domain and removes the ubiquitin moieties from target proteins. The deubiquitination function of the lid is carried out by DUBs namely Rpn11, Uch37, and USP14/Ubp6. To hold the 19S and 20S complexes together and to open the gate to the catalytic chamber, requires high energy nucleotides [99]. Specific functions of RP require energy, e.g. for the preparation and the translocation of target proteins into CP for degradation [101, 108].

1.7. Ubiquitin

Ubiquitin is a relatively small crucial protein of 76 amino acids (8.5 kDa), originally identified in the calf thymus, it is ubiquitously expressed in all eukaryotic cells ranging from yeast to mammalian cells [109, 110]. Ubiquitin either translated as polyprotein of tandem repeats (ubiquitin-B, ubiquitin-C) or like a single ubiquitin with an unrelated tail extension (ubiquitin A-52-residue ribosomal protein (UBA52), ribosomal protein S27A (RPS27A)) ([109-111]. Ubiquitin is both a nuclear and cytosolic protein. In the cytosol, it is involved in ATP-dependent non-lysosomal protein degradation whereas, in the nucleus, it is attached to H2A and regulates the chromatin structure and transcriptional activity [111]. Ubiquitin monomers are generated upon

the proteolytic process of precursor proteins by ubiquitin hydrolases. The generated ubiquitin monomers enter the ubiquitin pathway [112]. Though the ubiquitin is small, it has many well-studied residues to facilitate the interaction with ubiquitin binding domains and DUBs. These residues are hydrophobic and made up of Leu8, Ileu44 and Val70, hydrophobic patches located on β-sheets of ubiquitin and crucial for interactions with ubiquitin binding domains [113]. Apart from these patches, ubiquitin also contains flexible loops and TEK boxes. All of these are proven to be involved in protein-protein interactions. The C-terminal end flexible group is required to cleave ubiquitin by DUBs and distinguish ubiquitin from other ubiquitin-like modifiers such as SUMO [86, 114]. In understanding the effective mechanism of ubiquitylation, the non-covalent interactions between ubiquitin and ubiquitin domains of several proteins are vital. The ubiquitin binding domains are present in the regulatory proteins and other proteins participating in DNA damage repair and endocytosis where ubiquitylation results in non-proteolytic action and functions as assembly and signaling other proteins. The 26s proteasome also contains these ubiquitin-binding domains where they enable the binding of polyubiquitinated proteins to the proteasome for degradation. The ubiquitin binding domains present in the ubiquitylation cascade, help to bind of E2-ligases onto a ubiquitinated target and enables further ubiquitylation process [115, 116].

A group of several proteins, which are alike ubiquitin are called ubiquitin-like proteins (Ubls). The process of Ubls conjugation onto substrates is similar to the ubiquitination process. For example, the Ubl Nedd8 has 58 % similarity to the ubiquitin sequence and is involved in cell cycle control [117, 118]. The conjugation process of Nedd8 onto a target protein is called *neddylation*. Cullin family proteins are well-studied Nedd8 target proteins [119].

The intracellular ubiquitin levels are maintained by synthesis, recycling, and degradation. The former depends on the DUBs since they remove ubiquitin from their substrates and are recycled in the cell. The free cellular ubiquitin levels are somewhat controlled by ubiquitin itself and by DUBs [120, 121].

Ubiquitin activation takes place in two steps, which requires ATP. Ubiquitin activation is carried out by the ubiquitin-activating enzyme-E1. The first step results in the formation of ubiquitin

adenylate intermediate. In the second step ubiquitin transferred on to E-1 catalytic cysteine residue, with the production of AMP [122]. The loaded E-1 thus carries both adenylate and thioesters. The E-2 conjugation results in trans-thio-esterification between E1- and E2 (the ubiquitin transfers from E-1 to E-2). Finally, the nucleophilic effects on the E-2 ubiquitin thioester conjugated by the Lysine residue on the substrate or the free amino terminus of the target protein result in the formation of iso-peptide bonds called ubiquitination [123].

1.8. Ubiquitin-activating enzyme-E-1

The human genome encodes two E-1 ubiquitin-activating enzymes named as Uba-1 and Uba-6 [124]. As the name suggests, E-1 ubiquitin enzyme activates ubiquitin and starts the first step in the ubiquitination process. As described above, the ubiquitin activation by the E-1 enzyme is a two-step process, involving adenylation and formation of a thioester bond. The reaction starts with the activation of free ubiquitin by using its C- terminal glycine in an ATP dependent process. This results in ubiquitin adenylation and PPi release. In the second step, the adenylated ubiquitin binds to the E-1 cysteine residue and forms a covalent thioester linkage and then an AMP is released [122, 125]. The E-1 contains three structural elements; namely an adenylation domain, which binds to ubiquitin and ATP, a catalytic domain and the ubiquitin-fold domain, which binds selectively to the E-2 enzyme. The fundamental properties of ubiquitin-activating enzyme E-1 are recognition of free ubiquitin and its activation, and furthermore, selection of E-2 enzyme and subsequent ubiquitin charging on to E-2 (E2-ubiquitin) [126].

1.9. Ubiquitin-conjugating enzyme-E-2

At least 38 ubiquitin-conjugating enzymes (UBCs) are identified in humans and all of these enzymes have conserved ubiquitin-conjugating domains and catalytic cysteine residues. These catalytic cysteine residues bind to ubiquitin-activating enzyme-E1. E-2 binds to E-1 when E-1 contains the activated ubiquitin, then the thioestered ubiquitin transfers to the catalytic cysteine of E-2 from E-1 through another thioester bond. All the E-2s which are categorized so far bind to E-3s with loop-1, 2 and the N- terminal α-helix on the surface of E-2. Any sequence changes of these motifs affect the E-3 binding specificity. When a single E-2 acts together with multiple E-3s, the E-2 residues participating in the recognition of E-3 are not identical. For

example, UBE2N uses Arg6 and Lys10 to identify residues upstream of the rING domain of the E3 TRAF6, however, Arg7 and Lys10 of UBE2N intervene its connection to the Ubox of C terminus of HSC70 interacting protein (CHIP; also called as STUB1) [127]. It is suggested that E-2 enzymes may contribute to substrate recognition with or without the combination of E-3 ligases thus facilitate substrate specificity to the whole cascade [128].

1.10. E-3 Ubiquitin Ligases

Ubiquitination takes place when an E-3 ligase binds to both E-2 thioesterified ubiquitin (E-2~ ubiquitin) and the target substrate, forming a chain. Then, the ubiquitin is transferred to the target substrate from E-2 either directly or with the help of E-3 ligases, through a covalent ubiquitin thioester intermediate. The ubiquitination specificity is described by the pairing of E-2 enzymes to substrates. E-3 ligases are categorized based on their two catalytic domains, in these two; one domain facilitates the association with E-2 enzymes and another one is in-charge of precise substrate recognition. In eukaryotes, E-3 ligases are broadly classified into two classes HECT domain and RING domain based on the uniqueness of the domain involved in E-2 enzyme recognition. The human genome encodes more than 600 E-3 ligases but the representation between them is not equal, around 600 genes encode for RING-domain-E-3 ligases while only 30 genes encode for HECT-domain E-3 ligases [124].

1.10.1. HECT-E-3 ligases

The HECT-E-3 ligases are the first family of E-3 ligases discovered in 1995[129]. The HECT-E-3 ligases are found in all eukaryotes; yeast cells express 5 HECT-E-3s, whereas the human genome encodes for around 30 HECT-E-3s [130]. The HECT-domain expected to have an enzymatic activity, during the catalytic process, it forms a thioester-linked intermediate with activated ubiquitin, before catalyzing the covalent attachment of ubiquitin to target substrate proteins. The HECT domain is the catalytic domain and N-terminus ends of HECT-E-3s and defines substrate specificity [129, 131]. HECT-E-3s are classified into three subfamilies based on their amino acid sequence of motifs/regions present in the N-terminus end. They are Nedd4/Nedd4 like E-3s, HERC-E-3s harboring RLDs and other HECT-E-3s [129, 131-134]. Nedd-4/Nedd4 like E-3s contains WW domains [132], whereas HERC-E-3s harboring RLDs contains HECT and RCC like

domains [133, 134] and other HECT-E-3s do not have any WW or RLD domains [129, 131]. The HECT domain mainly facilitates the interaction with Ubch5 and a Ubch7 subgroup of E-2 ubiquitin-conjugating enzymes [135, 136], which results in the formation of a thioestered ubiquitin complex through the conserved cysteine residue. In the presence of E-2s, the capability to form a thioestered ubiquitin complex is essential for the ubiquitination of substrates. HECT-E-3s catalyzes the final attachment of ubiquitin to substrates as well as to ubiquitin molecules in order to form polyubiquitin chains [129, 137-139]. The HECT-E-s activity is regulated by two levels, an association of HECT-E-3 with its substrate protein and another level is related to the catalytic activity of HECT domain that includes interaction with E-2 ubiquitin-conjugating enzyme [138].

1.10.2. Ring E-3 Ligases

The majority of the E-3 ligases and the RING E-3 ligases contain a RING domain, which was first defined as a cysteine-rich sequence in 1991 by Freemont and his colleagues [140]. RING E-3s serve as scaffold enzymes on which the E-2 enzyme and the substrate interact for the catalytic process [67]. The RING domain has approximately 40-60 amino acids and coordinates with two zinc (Zn) ions.

The RING domain, also known as the C3HC4 motif has a basic sequence Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X_2-Cys-$X_{(4-18)}$-Cys-X_2-Cys, where X refers to any amino acid and the bracket represents an intervening sequence length. In the RING domain structure, one Zn atom binds with four cysteine residues and another Zn binds with three cysteine residues and one histidine. The three-dimensional structure of the RING domain demonstrates the conserved histidine and cysteine residues binding with two Zn atoms inside the core of the domain [124, 141, 142]. Among several reported variations, cysteine and histidine residues are swapped or the cysteine residue is substituted by an alternative residue to coordinate Zn. For example, cysteine is replaced by asparagine in Rbx or Roc1[143].

RING-E-3s are classified based on their function; they exist in monomers, dimers, and multi-subunit complexes. The majority of the RING E-3s are multi-subunit E-3s and Cullin-Ring Ligases (CRL) known to be the largest class of ubiquitin ligases. Due to huge diversity in substrate-receptor subunits of CRLs, there are hundreds of different CRLs that are possibly present in

eukaryotes and shows CRLs as important mediators of post-translational protein regulation and a well-studied family of RING-E-3s. As described in figure 8, the CRLs are scaffold proteins (CUL1, 2, 3, 4A, 4B, 5 & 7) which serve as a binding platform for other proteins [144], substrate binding proteins (like Skp1) and RING domain proteins (ROC-1/RBX-1, RBX-2, and HRT-1). Substrate binding proteins contain adaptor proteins like (F-box, SOCS/BC box, and BTB)[145]. Generally, the CRLs which contain CUL1 as scaffold protein is called SCF complexes [145] [146].

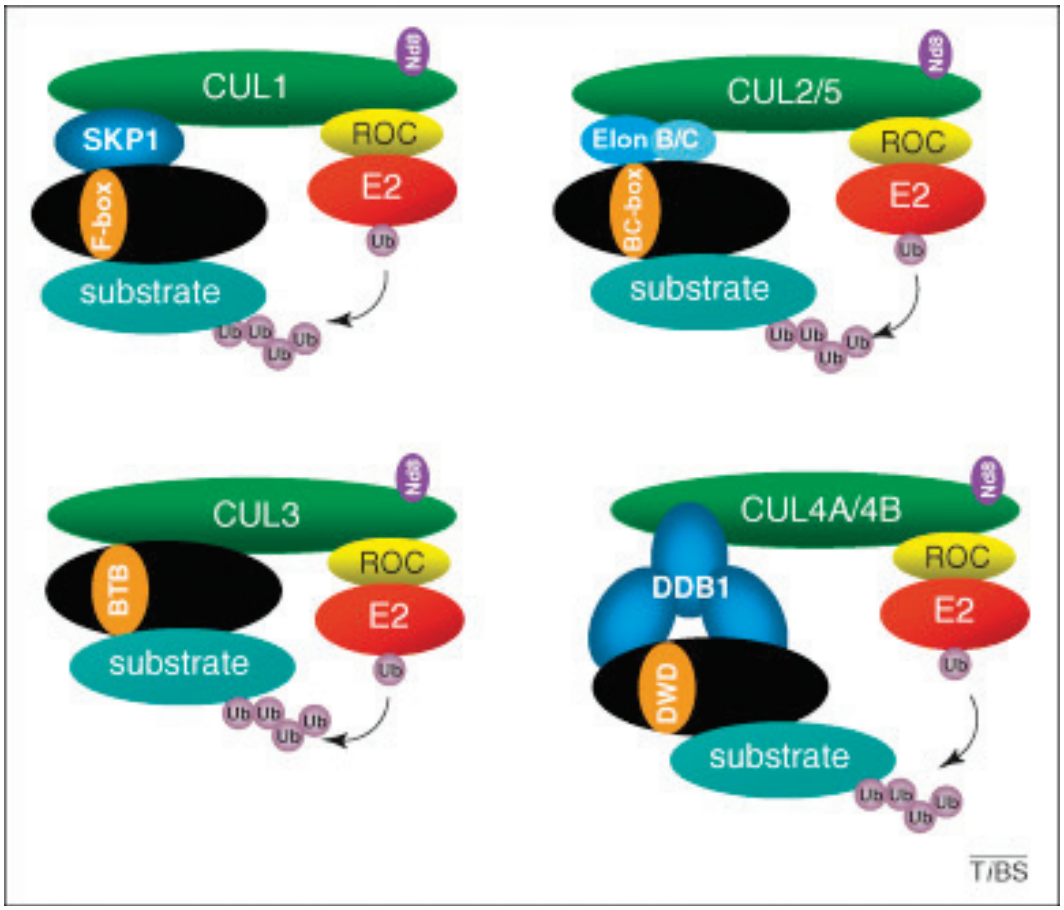


Figure 8. Assembly of cullin-RING ubiquitin ligases (CRLs) *(taken from [147]). The cullin–ROC family of E3-ligases controls the ubiquitylation of several substrates by assembling into multiple distinct E3 ligases. Each cullin uses a modular assembly to recruit different substrates to a common catalytic core by varying its substrate receptor.*

1.10.3. SCF-E-3 ligases and their function

The Skp-1 Cullin F-box complex (SCF) E-3 ligase family is the majority among the 600 E-3s [148-150]. As shown in figure 9, the SCF complex contains Cullin-1 as a scaffold protein, the RING domain-containing protein Roc-1/RBX-1, Skp1 adaptor protein, and the variable F-box protein, which recruits the target substrate and links the target substrate to SCF complex through Skp1 [148, 151]. SCF-E-3 ligases are mainly involved in cell-cycle regulation. Anaphase Promoting Complex (APC) is another example for CRLs, similar to the SCF complex in structure and contains 13 sub-units [152]. Even though both complexes have structural and biochemical similarities, they differ in cellular functions. The APC complex controls cell- cycle progression, such as S phase entry, G1 phase, and G-2/M phase exit. Whereas, the SCF complex mainly regulates G1, S, and early M phase [153]. In order to promote the S phase entry during cell cycle progression, the SCF complex degrades cyclin-dependent kinase inhibitors (CKIs) and G1 cyclins [154]. The variable F-box proteins play a major role in substrate recruitment to the SCF complex.

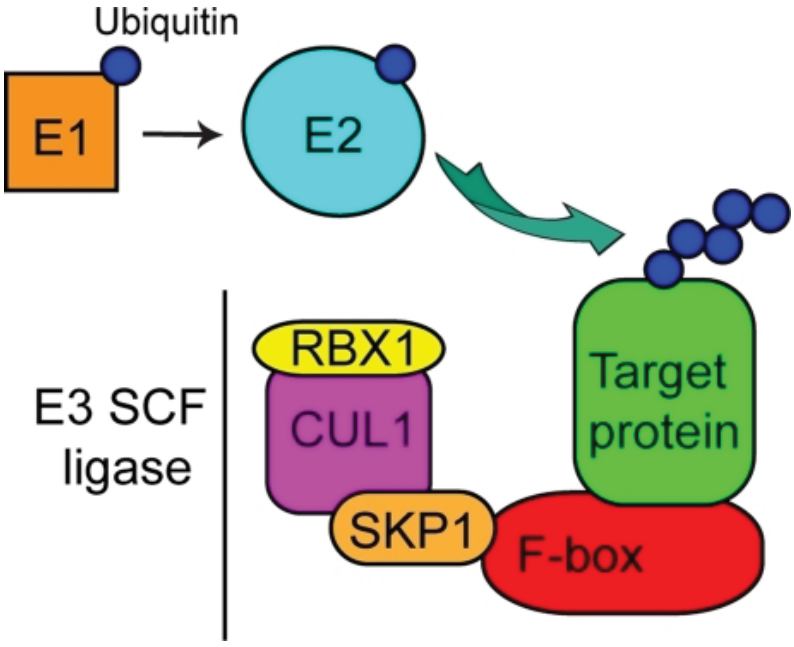


***Figure 9**. **The SCF complex** (taken from [155]). E1 transfers ubiquitin to E2, then ubiquitin conjugates to a target protein associated with the F-box protein within the E3 SCF multi-protein complex. The SCF complex contains the RING-domain protein RING-box 1 (RBX1), Cullin 1 (CUL1), S-phase-kinase associated protein 1 (SKP1), and a protein harboring an F-box domain, that directly binds SKP1, F-box- domain binds to specific substrates to be ubiquitinated and target them to the E3 ligase multimeric complex that is bound to the F-box domain.*

1.10.4. F-box Proteins

In humans, more than 60 different F-box proteins are present, but only a few of them are well characterized for their target substrate [156, 157]. F-box proteins are further classified into three subclasses based on the additional protein-protein interaction motifs; the FBXW, FBXL and FBXO proteins [158]. FBXW proteins contain WD40 repeats. Well-characterized proteins from this family are β-TRCP1, FBXW7 also called FBXW11 and β-TRCP2 known as FBXW12. The second family is FBXL proteins containing Leucine-rich repeats and comprises 22 members including a well-known protein, Skp2 also known as FBXL1. The third one is FBXO family called as F-box Only (FBXO) proteins, containing an F-box domain with or without any other protein-protein interaction motif. Other F-box proteins have additional domains like leucine zippers, zinc fingers, cyclin domains, ring fingers, proline-rich regions, and tetratricopeptide (TTR) repeats [154, 158, 159].

F-box protein recognizes substrates once they are phosphorylated at particular sites [149]. Mostly, F-box proteins recognize their substrates by targeting specific degrons. Degrons are short and well-defined motifs present on the substrates [160, 161]. Furthermore, F-box protein's interaction with target substrates requires appropriate post-translational modification of substrates [162]. For example, FBXW7 substrates characteristically possess a conserved CDC4 phosphodegron sequence known as CPD sequence (Leu)-X-pThr (or pSer)-Pro-Pro-X-per (or pThr, Glu or Asp) where X represents any amino acid. In order to be phosphorylated, CPD degrons are recognized by the FBXW7 to ubiquitinate the substrates [162, 163]. F-box proteins can also recognize other forms of degron modifications; such as glycosylation or addition of mannose oligosaccharides substrates. FBX2 specifically recognizes N- linked high mannose oligosaccharides and leads to ubiquitination. Precursor β1 integrin is the substrate for FBX2 [164]. FBX6b interacts with N-glycated T-cell receptor α chain (TCRα) which is a substrate in the endoplasmic reticulum-associated degradation (ERAD) pathway [165].

The F-box proteins are present both in the cell nucleus and the cytoplasm [166, 167]. The identification of sub-cellular localization of F-box proteins is very crucial; however, studies on localization are limited. [154].

1.10.4.1. F-box only protein 28 (FBXO28)

FBXO28 is an F-box protein contains approximately 40 amino acids and localized in the nucleus (nuclear F-box protein). FBXO28 is an evolutionary, conserved (FBXO28 homologs are present in vertebrates and *Drosophila),* and a cell cycle directed protein. Substrate recruiting domain of SCF-complex FBXO28 plays a crucial role in recruiting proteins for degradation or localization for several cellular processes including cell proliferation, cell cycle progression, and transcription of specific genes [167, 168]. FBXO28 expression levels vary across the cell cycle but elevated levels observed during G2 to M transition phase. The localization of FBXO28 is controlled during the cell cycle; endogenous FBXO28 is localized in the nucleus during interphase and during mitosis, it localizes in the area of the mitotic chromosomes. Being a nuclear protein FBXO28 is crucial for appropriate mitogenic progression as it interferes with mitotic progression during metaphase to anaphase transition [167, 168].

Additionally, recent research demonstrated that FBXO28 plays a crucial role in maintaining genomic stability. The depletion of FBXO28 results in multinucleation in cells. A time-lapse video microscopy study on HeLa cells revealed that cells with depleted FBXO28 have prolonged mitosis. In addition, mitotic defects (lagging chromosomes, multipolar spindles, and multinucleated cells) were also observed in FBXO28 depleted cells. FBXO28 interacts with Type IIα topoisomerases (Topo IIα) [167], which involves the separation of daughter strands of DNA during replication process and crucial efficient chromosome condensation [169]. Defects in Topo IIα activity resulted in polyploidy cells and defects in chromosome segregation. Altogether, Topo IIα is crucial for cell cycle progress [170, 171]. FBXO28 interacts with Topo IIα upon phosphorylation by CK2 thus regulating the decatenation activity of Topo II*α* and by playing a crucial role in genomic stability [167].

It has been reported that FBXO28 function depends on its phosphorylation. To become functionally active FBXO28 needs to be phosphorylated by its upstream regulators; Diana et al. reported that a phospho-deficient $SCF^{S344A\text{-}FBXO28}$ complex was not efficient in catalyzing MYC ubiquitination compared to wild-type $SCF^{WT\text{-}FBXO28}$ complex, suggesting that SCF^{FBXO28} intrinsic ubiquitin ligase activity is switched on by CDK1/2. Cyclin-dependent kinases CDK1/2 phosphorylate FBXO28 at S344 [168, 172]. Phosphorylated FBXO28 levels are minimal during G1

phase but peaked in S, G2/M phase which is similar to total FBXO28 levels. The phosphorylated FBXO28 regulates MYC regulated gene expression by ploy ubiquitination. The non-proteolytic ubiquitination of MYC by FBXO28 is required for MYC driven transcription [168].

It is clearly known that dysregulation of UPS contributes to multiple disorders and diseases. Such as several cancers, neurodegenerative disorders, and metabolic disease like diabetes [173, 174]. UPS dysregulation in diabetes resulted in β-cell death and insufficient insulin secretion [174]. Recent research also shows, based on microarray analysis FBXO28 gene expression levels are downregulated in T2D. FBXO28 plays a crucial role in several cellular processes and diseases but information regarding FBXO28's regulation and function in diabetes and pancreatic β-cells are not available.

1.11. Deubiquitination

Ubiquitination is a reversible process; the removal of ubiquitin moieties from a ubiquitin-conjugated target protein is known as deubiquitination and relies on deubiquitinating enzymes (DUBs). The DUBs are proteases that recognize ubiquitin-tagged proteins and cleave the ubiquitin tag and thus remove the monoubiquitination or alter the polyubiquitination of the target protein. Similar to ubiquitination that controls protein functions, deubiquitination regulates the protein destiny and its function [175]. Like ubiquitination, deubiquitination is also a closely regulated process that regulates several cellular processes, including cell cycle regulation, DNA damage repair, gene expression, proteasome and lysosome dependent protein degradation, gene expression, microbial pathogenesis [176]. Apart from deubiquitination, DUBs play an important role in maintaining the homeostasis of the free ubiquitin pool in cells. Ubiquitin genes create polyubiquitin protein precursors, specific DUBs such as USP5/IsoT cleave monoubiquitin from the precursor. Also, DUBs maintain the ubiquitin homeostasis in a cell by recycling the ubiquitin moieties from degraded proteins. In order to recycle the ubiquitin from targeted proteins, the proteasome itself contains specific DUBs, such as USP-14, UCHL-5, and POH-1, which hydrolyze the chains prior to degradation of the target proteins [177, 178]. Only a few enzymes are involved in preserving the stable pool of monoubiquitin in cells. Most of the

DUBs regulate protein ubiquitination events directly. Classically, protein ubiquitination results in protein degradation, and deubiquitination causes the stability of proteins. DUBs have high specificity towards the substrate selection [179]. Apart from their active site domain, the majority of DUBs comprise additional domains which regulate protein-protein interactions and substrate recognition [176].

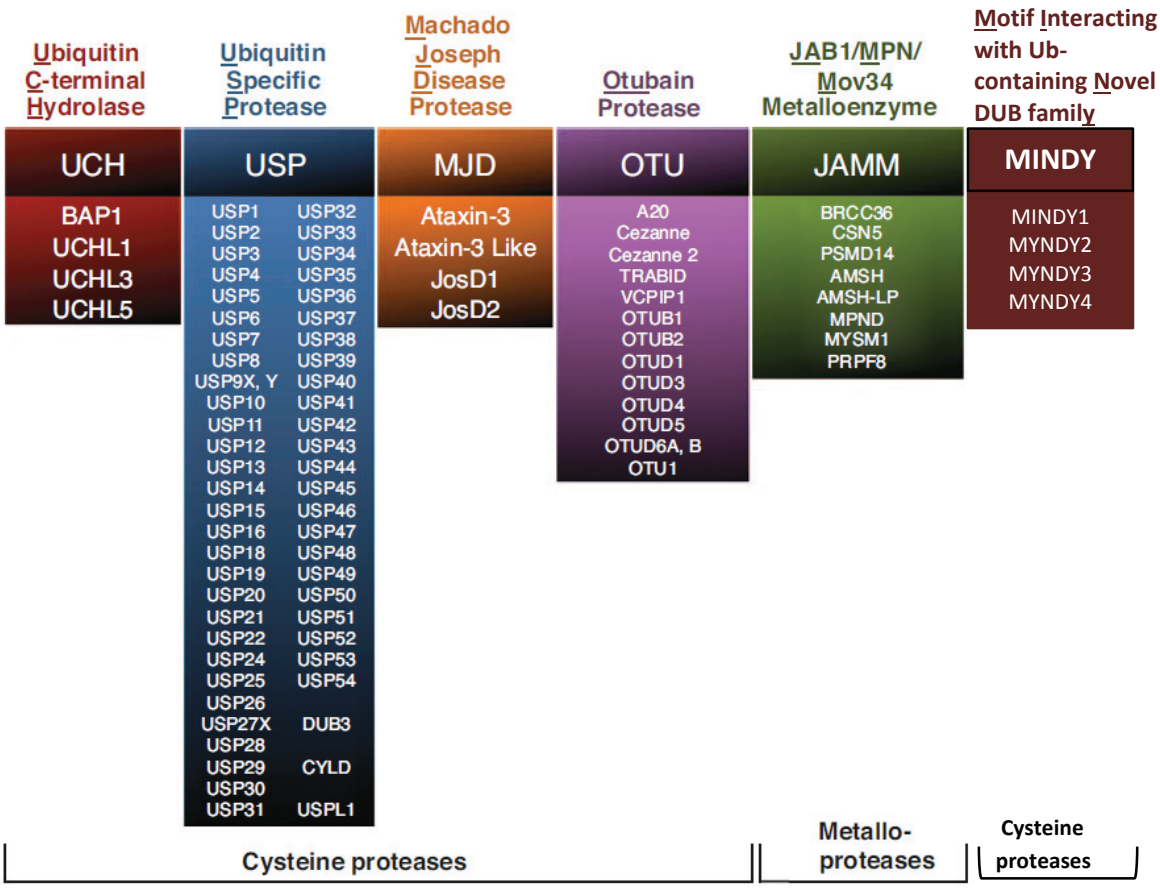


Figure 10. Deubiquitinating enzymes (DUBs) *(taken from[181]) DUBs are classified into ubiquitin carboxyl-terminal hydrolases (UCHs),ubiquitin specific proteases (USPs), Machado Joseph disease (MJD) proteases, otubain (OTU) proteases, JAB1/MPN/Mov34 metalloenzyme (JAMM) subclasses and motif interacting with Ub-containing novel DUB family (MINDYs). All are cysteine proteases except JAMM DUBs, which are zinc-dependent metalloproteases.*

The human genome encodes around 100 DUBs. As described in figure 10, DUBs are categorized into six families: ubiquitin COOH-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), Machado Joseph Disease proteases (MJDs), ovarian tumor proteases (OTUs), the

JAB1/MPN/MOV34 family (JAMMs) and the motif interacting with Ub-containing novel DUB family (MINDYs) (Figure 10). USPs, OTUs, UCHs, MJDs, and the recently recognized MINDYs are cysteine proteases, whereas the sixth family, JAMMs, contains zinc metalloproteases [178, 180].

1.11.1. USP family of DUBs

The USPs are the major sub-family of DUBs; humans have more than 50 USPs whereas Yeast consists of 16 USPs. The domain (USP) structures of six USP DUBs show that the USP domain fold is extremely conserved [176].

1.11.1.1. Structure and function

USPs contain a catalytic domain of at least 400 amino acids with a conserved cysteine and histidine-containing motif extensions; these two motifs are required for their activity. Four additional motifs are also conserved in sequence; however, they are not mandatory [182, 183]. The USP domain can be divided into three sub-domains; finger, palm, and thumb, which collectively resemble like a right hand. Only one CYLD DUB lacks the finger sub-domain. Structural studies of USP domains bound to ubiquitin and ubiquitin-based inhibitor studies reveal that the ubiquitin C-terminus sits in a cleft situated between the thumb and the palm sub-domains. Furthermore, the ubiquitin globular portion collaborates with the finger domain [176, 183-185]. The USPs containing extensions on one or both sides of the core domain play a crucial role in targeting sub-cellular structures and protein-protein interactions[182].

Ubiquitin is connected to different proteins by means of an isopeptide linkage. Certainly, USPs that are characterized to date exhibit isopeptidase action against short polyubiquitin chains linked through Lys-48 in vitro [186, 187]. Such isopeptidase activities might act at distinct steps within the pathway. These activities have been noted as editing or recycling functions [182]. Editing results in reversal of the ubiquitination impact. Recycling refers to isopeptidase activities that actually regenerate free ubiquitin once the ubiquitination process is completed. Even though theoretically beneficial, the capabilities are not completely different as editing also discharges ubiquitin that can be recycled.

Among all the USP DUBs, Ubiquitin-specific protease-1 (USP1) is one of the well-characterized USP and plays a crucial role in tumorigenesis and DNA damage repair. USP1 is first characterized as a key player in DDR [188].

1.11.1.2. Ubiquitin-specific protease-1 (USP1)

As mentioned earlier USP1 is one of the well-studied human DUBs, which plays a critical role in the cellular response to DNA damage. USP1 protein levels, localization, and activity are influenced and modulated by several mechanisms, which includes protein-protein interactions, auto cleavage/degradation, and phosphorylation, ensuring that USP1 function is finished in a well-regulated spatiotemporal manner. Significantly, the USP1 expression is dysregulated in human disease; such as in some cancers [189]. This makes USP1 a potential target in medical research or in disease-related area.

The USP1 gene encrypts a protein, which consists of 785 amino acids with an expected molecular weight of 88.2 kDa. As described in figure 11, USP1 contains a USP domain with N-terminal Cys box motif which contains catalytic residue Cys90 and C-terminal His box, which contains catalytic residues His593 and Asp751 [189-191].

Figure 11. USP1 domain structure *(taken from[189]). The USP1 protein contains Cys box on amino-terminal and His bon carboxy-terminal.*

USP1 regulation and Function

USP1 expression levels, catalytic activity and access to substrates are regulated by numerous mechanisms to make sure that USP1 activity is appropriately controlled. USP1 gene transcription is regulated in a cell cycle-dependent manner. During G1 phase, USP1 mRNA levels stay low and reach to top level through the S-phase [192]. USP1 cell cycle-dependent expression levels are controlled at the protein level by proteasomal degradation. As shown in figure 12, the USP1

295-342 region contains a destruction motif (degron) that is essential for Anaphase Promoting Complex/CyclosomeCdh1 (APC/CCdh1)-dependent degradation of USP1 throughout G1 [193].

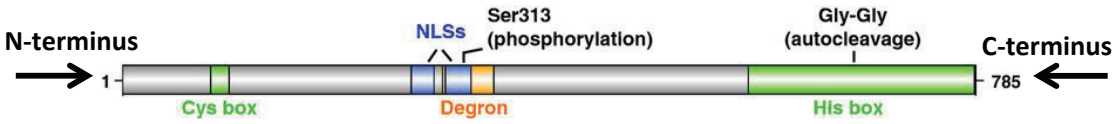


***Figure 12**. **USP1 regulatory motifs** (taken from[189]). The USP1 protein showing the position of sequence motifs, which contribute to the regulation of USP1 levels, localization and function. The regulatory motif of USP1, the degron (orange) mediates APC/C^{Cdh1}-mediated degradation of USP1 and two NLSs (blue) mediate the import USP1/UAF1 complex into the nucleus. The diglycine motif (Gly-Gly) is the site for USP1 autocleavage, and the Ser313 residue is the site for Cdk-mediated USP1 phosphorylation.*

Apparently, the foremost crucial event within the regulation of USP1 deubiquitinase activity is its association with UAF1 [194]. The UAF1 protein consists of WD40-repeats that additionally binds to and regulates two other DUBs, USP12 and USP46 [195]; however, its best-characterized role is as a co-factor of USP1 with regards to a USP1/UAF1 complex. The enzymatic action of USP1 alone is incredibly low and is significantly improved after its binding to UAF1 [194]. Despite the fact that the molecular basis for UAF1-mediated USP1 activation remains to be completely comprehended, recent in vitro studies using artificial substrates assist an allosteric mechanism, including UAF1-induced conformational changes in the USP1 active site [191].

Other than increasing its catalytic activity, UAF1 additionally stabilizes USP1 [194] and mediates its interaction with USP1 targets such as ub-FANCD2 and ub-PCNA [196]. In order to reach the nuclear substrates, USP1/UAF1 complex initially calls for the nuclear import that is mediated with the aid of two nuclear localization signals (NLSs) in USP1 [197]. At that point, a SUMO-like domain (SLD2) in UAF1 intervenes the precise targeting of the complex to ub-FANCD2 through direct binding to FANCI [196] also, ub-PCNA through direct binding to the PCNA partner ATAD5/ELG1 [198].

1.11.1.3. USP1 regulated pathways

USP1 acts as a controller of several vital steps within the DNA damage response pathways specifically, in Fanconi anemia (FA) pathway [192], and translesion synthesis (TLS) process [199].

a) Fanconi Anemia (FA) pathway

The well-studied USP1 function is the regulation of various crucial steps in DNA damage response, primarily in the FA pathway [189]. USP1 physically interacts with a key member of FA pathway FANCD2 and colocalise in chromatin after DNA damage [192]. FA is an atypical hereditary disease characterized by congenital abnormalities, progressive bone marrow failure, hypersensitivity to DNA crosslinking agents, genomic instability and a multiplied susceptibility to cancer [200]. Mutations in a minimum of fifteen genes cause FA. The resulted products of those genes are dynamic within the DNA repair pathway which fixes interstrand crosslinks (ICL), a DNA lesion which leads to polymerase stalling. In order to correct this kind of genomic damage, eight FA proteins gather in a nuclear complex with ubiquitin E3 ligase activity, named as FA core complex, which monoubiquitinates different FA proteins, FANCI and FANCD2 at the site of the damage. Thus, monoubiquitinated FANCI (ub-FANCI) and FANCD2 (ubFANCD2) function as a platform to enlist the particular nucleases, polymerases and other DNA repair proteins that complete the following strides of ICL correction [201]. Figure 13 shows, USP1 deubiquitinates each of ub-FANCI [202] and ub-FANCD2 [192], therefore, reverting the crucial event within the activation of the FA pathway. USP1mediated deubiquitination of FANCD2 and FANCI is vital for the proper function of the FA pathway, as proven by the perception that USP1 gene knockdown in murine models or DT40 chicken cells restates numerous phenotypical aspects of FA pathway, together with hematopoietic defects and hypersensitivity towards DNA crosslinking agents [189].

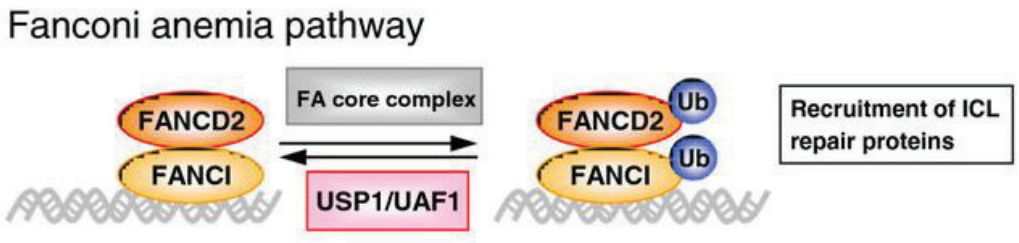


***Figure 13**. **Fanconi anemia pathway** (taken from[189]). FA core complex monoubiquitinates both FANCI and FNACD2 proteins. The monoubiquitination of FANCI and FNACD2 recruits other proteins which repair DNA interstrand crosslink lesions (ICL). The USP1/UAF-1 complex deubiquitinates the FANCI and FNACD2 proteins.*

b) Translesion synthesis (TLS)

Translesion synthesis is another DNA damage repair process regulated by USP1, which supports the role of the DUB in the DDR. PCNA (Proliferating Cell Nuclear Antigen) is the crucial substrate for USP1 in TLS [199]. As showed in figure 14, upon DNA damage, which slows down the movement of the replication fork, PCNA is monoubiquitinated by the Rad18 E3 ligase at lysine 164 [203]. The monoubiquitinated PCNA (ubPCNA) allows the recruitment of unique TLS polymerases, which could pass the lesion [204]. PCNA could even be monoubiquitinated by the $CRL4^{Cdt2}$ E3 ubiquitin ligase complex without any DNA damage [205]. TLS polymerases have a lower constancy than replicative polymerases, and may hence lead to a higher mutagenesis rate. Through reverting PCNA monoubiquitination [199], USP1 avoids the recruitment of TLS polymerases, and may for this reason help preserving genome stability [206].

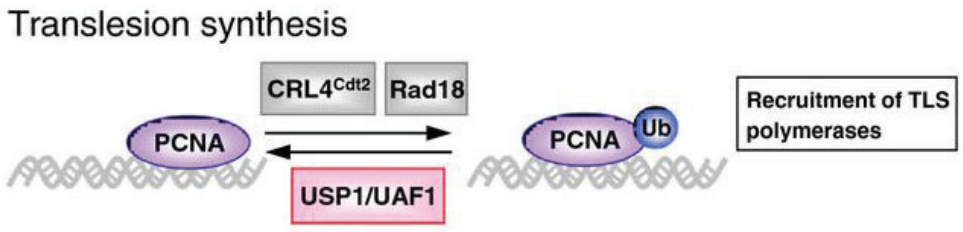


Figure 14. Translesion synthesis pathway *(taken from[189]). Rad18 or $CRL4^{Cdt2}$ E3 ubiquitin ligase complex monoubiquitinates PCNA. The monoubiquitinated PCNA recruits TLS DNA polymerases to bypass DNA lesions other proteins which repair DNA interstrand crosslink lesions (ICL). The USP1/UAF1 complex deubiquitinates the PCNA thus prevents the recruitment of TLS DNA polymerases.*

USP1 contributes to the repair of double-strand DNA breaks through homologous recombination. The molecular mechanism underlying this activity is not clear; however, it seems to involve in the suppression of nonhomologous end-joining pathway [207]. Other than these DNA damage repair-related capacities, USP1 deubiquitinates and stabilizes three proteins from the family of inhibitors of DNA binding (ID) proteins, specifically ID1, ID2, and ID3 [208]. ID proteins are expressed in the course of development in many undifferentiated and proliferating cells. ID proteins are negative controllers of basic helix-loop-helix (bHLH) type transcription factors that bind to DNA and promote the differentiation of numerous cell varieties [209]. Through the deubiquitination of ID proteins, USP1 contributes to forestall the

bHLH mediated differentiation and therefore maintain stem-cell characteristics in osteosarcoma cells [208].

1.12. The UPS in pathophysiology and metabolic diseases

In physiological situations, the proteasome degrades ubiquitinated proteins once their unique lifespan has been come to an end [160]. In instances of oxidative and endoplasmic reticulum (ER) stress, the UPS eradicates misfolded proteins [160, 210]. UPS dysfunction is mainly implicated in the development of neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, Huntington disease, prion-like lethal disorders and is also involved in the Angelman's syndrome, Liddle syndrome and several cancers [173].

Cellular and organismic metabolism affects proper UPS regulation. Hyperglycemia-induced oxidative stress and several aging processes affect the normal function of UPS quality control in retinal cells and kidney. This accounts for the pathogenesis of Age-related Macular Degeneration (AMD), diabetic retinopathy and diabetic nephropathy [211-214]. Also, it was found that a decrease in vascular proteasome activity serves as a major contributing factor in the development of cardiovascular atherosclerosis [215].

UPS is vital in the regulation of pancreatic β-cell function and thus in the regulation of glycemia. Inhibition of proteasome activity leads to misfolding and aggregation of glucokinase, and significantly reduced glucose-induced insulin secretion [174]. In pancreatic β-cells, the harmony between protein synthesis, folding, and degradation are vital to keeping up proper glucose-induced insulin secretion [210]. Human, mouse and rodent pancreatic islets and MIN6 β-cells incubated with a proteasome inhibitor indicated a drastic reduction in glucose-stimulated insulin secretion, while the insulin content stayed unaltered [216-218]. Similar to insulin biosynthesis [216], UPS also regulates proteins that contribute to insulin secretion pathway which include ATP-dependent potassium and voltage-dependent calcium channels [210, 218, 219]. However, the impact of the UPS on other significant regulators of the insulin secretion apparatus is far less known but is of emerging interest [210].

As a contrary, it is also known that UPS plays a vital role in the degradation of IRS protein components. This alteration in the protein turn-over by UPS contributes to the major defects in diabetes and insulin resistance [220]. Currently, research is carried out to explore the key components of UPS and its role in the pathogenesis of several metabolic disorders. Hence, UPS is exploited to be a major therapeutic target for several diseases.

Aim of the Thesis

Pancreatic β-cells secrete the exact amount of insulin to maintain glucose homeostasis. In diabetes, β-cells fail to fulfill this physiological requirement. The loss of β-cell mass together with insufficient insulin secretion is the cause of the disease [221]. Identifying and establishing novel therapeutic targets in order to restore β-cell survival and function is a promising approach for the treatment of diabetes. The UPS pathway plays a crucial role in the regulation of multiple cellular processes such as proliferation, differentiation, and transcriptional regulation [222], in particular, β-cell maturation, survival and maintaining their phenotype [223-225]. The primary focus of my thesis is to investigate the role of the UPS component, E-3ligase SCF^{FBXO28}, and de-ubiquitinase USP1 in β-cell survival and function at normal physiological as well as in diabetic state. My major goal is to further understand the regulation and function of UPS components responsible for the molecular and cellular alterations in the process of β-cell failure in diabetes. I have used gene manipulations (knockdown/overexpression) and pharmacological inhibitors in human islets as well as in rodent β-cells to investigate the effectiveness of ubiquitin-proteasome components (FBXO28 & USP1) in β-cell survival and function. Cell death markers cleaved caspase-3 and cleaved PARP protein levels were used to analyse the β-cell death and survival upon gene manipulations and drug intervention. A well-established DNA damage response markers γ-H2AX and p-P53 (involved in DDR activation) were analyzed to understand the DNA damage response. Pancreatic β-cell functionality was determined by glucose-stimulated insulin secretion (GSIS) as well as by mRNA analysis of β-cell functional and maturation genes.

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2. Results

Manuscript 1

2.1. An SCFFBXO28 E3 ligase protects pancreatic β-cells from apoptosis

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An SCF^{FBXO28} E3 Ligase Protects Pancreatic β-Cells from Apoptosis

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Abstract: Loss of pancreatic β-cell function and/or mass is a central hallmark of all forms of diabetes but its molecular basis is incompletely understood. β-cell apoptosis contributes to the reduced β-cell mass in diabetes. Therefore, the identification of important signaling molecules that promote β-cell survival in diabetes could lead to a promising therapeutic intervention to block β-cell decline during development and progression of diabetes. In the present study, we identified F-box protein 28 (FBXO28), a substrate-recruiting component of the Skp1-Cul1-F-box (SCF) ligase complex, as a regulator of pancreatic β-cell survival. FBXO28 was down-regulated in β-cells and in isolated human islets under diabetic conditions. Consistently, genetic silencing of FBXO28 impaired β-cell survival, and restoration of FBXO28 protected β-cells from the harmful effects of the diabetic milieu. Although FBXO28 expression positively correlated with β-cell transcription factor *NEUROD1* and FBXO28 depletion also reduced insulin mRNA expression, neither FBXO28 overexpression nor depletion had any significant impact on insulin content, glucose-stimulated insulin secretion (GSIS) or on other genes involved in glucose sensing and metabolism or on important β-cell transcription factors in isolated human islets. Consistently, FBXO28 overexpression did not further alter insulin content and GSIS in freshly isolated islets from patients with type 2 diabetes (T2D). Our data show that FBXO28 improves pancreatic β-cell survival under diabetogenic conditions without affecting insulin secretion, and its restoration may be a novel therapeutic tool to promote β-cell survival in diabetes.

Keywords: pancreatic β-cell; diabetes; human islet; apoptosis; FBXO28; insulin secretion; NeuroD1; E3 ligase

1. Introduction

Insulin-producing pancreatic β-cells are key endocrine cells in regulating blood glucose levels and metabolic homeostasis. Progressive loss of β-cell function and/or mass, which is triggered by programmed cell death contributes to the pathogenesis of both type 1 diabetes (T1D) and type 2 diabetes (T2D) [1–3] Also, other mechanisms such as β-cell dedifferentiation [4] and defective β-cell proliferation and regeneration [5] are hypothesized as causes for the pancreatic β-cell insufficiency in diabetes. In T1D, β-cells are destroyed by the autoimmune attack of macrophages and T-cells through several deleterious mechanisms such as inappropriately high production of pro-inflammatory cytokines, chemokines, reactive oxygen species (ROS) and other apoptotic stimuli like the perforin/granzyme B and Fas/FasL systems [6]. In T2D, loss of functional β-cell mass is progressive and usually occurs when β-cells are no longer able to provide sufficient amounts of

insulin in response to a higher insulin demand under insulin resistance and metabolic pressure. Inappropriately elevated metabolic factors such as glucose, fatty acids and islet amyloid polypeptide (IAPP), together with inflammation induce progressive β-cell dysfunction and death by mechanisms such as endoplasmic reticulum (ER) and/or oxidative stress [7–11]. Consequently, inhibition of β-cell death and dysfunction represents a promising therapeutic approach for the β-cell-directed therapy of diabetes.

The cellular protein abundance and turnover is regulated by the intracellular degradation mechanism called the ubiquitin-proteasome system (UPS). The stability of proteins is controlled by several post-translational modifications, including ubiquitination [12]. By adding small protein ubiquitin to target proteins, they are usually marked for degradation by the proteasome or by the lysosome [13,14]. The intracellular protein degradation program mainly governed by UPS has an essential role in the cell cycle regulation, cell survival, and eradication of misfolded proteins. In pathological conditions, i.e., in major human diseases such as cancer, immunological and neurological disorders [13–15] as well as β-cell failure in diabetes [16–23], the UPS machinery is often de-regulated.

The ubiquitin conjugation to a substrate protein is coordinated by a series of enzymatic reactions; an E1 ubiquitin-activating enzyme initiates ubiquitination and transfers ubiquitin to an E2-conjugating enzyme, which then interacts with E3-ubiquitin ligase. E3 carries out the final step, the transfer of ubiquitin to the target protein by the formation of a covalent isopeptide bond between the substrate's lysine and the carboxyl-terminus of ubiquitin [13,24]. The Skp1-Cul1-F-box (SCF) protein ligase complex is one of the biggest and best described among the E3 ubiquitin ligase family. The SCF complex contains a catalytic core complex comprising of Skp1, Cullin1 and Roc1/Rbx1/Hrt1. F-box proteins are adaptor receptor subunits of this SCF complex and are responsible for recruiting protein substrates through phospho-specific domain interactions [25]. The F-box protein within the E3 ligase complex works as the scaffold connecting the ligase complex with the particular substrates by its F-box domain and substrate binding motif [25]. F-box protein 28 (FBXO28) is an important nuclear F-box protein with incompletely understood cellular functions. Recent research showed that FBXO28 is involved in cell cycle regulation and required for appropriate mitotic progression [26,27]. Loss of FBXO28 results in metaphase to anaphase progression delay, which then leads to several mitotic defects like lagging chromosomes, multipolar spindles and multi-nucleation [27]. A microarray-based analysis identified decreased expression of FBXO28 in human islets isolated from patients with T2D [16]. As the potential action of FBXO28 has never been investigated in diabetes and particularly in the pancreatic β-cell so far, we sought to determine the role of FBXO28 on pancreatic β-cell survival and function under diabetic conditions using clonal β-cells as well as isolated primary human islets from control and diabetic individuals.

2. Results and Discussion

2.1. Loss of FBXO28 Induces β-Cell Apoptosis

Dysregulation of the UPS machinery as well as of its components has been observed in β-cells/islets under diabetic conditions [16–23]. The gene expression of a number of UPS components including several F-box proteins such as FBXO3, FBXO11, FBXW12 and FBXO28 were highly changed in islets isolated from patients with T2D compared to healthy individuals according to previously published microarray-based transcriptome analyses [16]. As impaired β-cell survival is a key pathogenic hallmark of diabetic β-cells [3] and *FBXO28* gene expression level was suggested to be downregulated in T2D islets [16], we sought to investigate the effects of F-box family member, FBXO28, which is expressed in β-cells in the well-established clonal β-cell line INS-1E as well as in isolated human islets (Figure 1) on β-cell survival at basal conditions. FBXO28 protein level was reduced under in vitro treatments commonly used to mimic human diabetes in rodent INS-1E cells (Figure 1A–D), i.e., by elevated glucose concentrations (22.2 mM) and by the cytokine mixture of

interleulin-1β (IL-1β), and interferon γ (IFNγ). Consistently, human islets treated with the combination of high glucose and the free fatty acid palmitate as well as pro-inflammatory cytokines show profound down-regulation of FBXO28 protein (Figure 1E,F). In order to understand the physiological impact of such decreased FBXO28 expression under diabetogenic conditions, siRNA was used to knockdown FBXO28 in INS-1E cells. INS-1E cells were transfected with both small interfering RNA (siRNA) against FBXO28 (siFBXO28) or siScr (served as a transfection control). Loss of FBXO28 induced basal β-cell apoptosis, as depicted by increased caspase-3 and PARP cleavage, both well-established markers of apoptosis (Figure 1G,H). In order to test whether the functional F-box domain (which links F-box proteins to the SCF complex via binding to Skp1) in FBXO28 is required for β-cell survival, we transfected the F-box domain-deleted FBXO28 mutant (ΔF-FBXO28) into INS-1E cells. Consistent with our results on FBXO28 depletion, overexpression of defective ΔF-FBXO28 induced capase-3 and PARP cleavage in INS-1E cells indicating that functional FBXO28 is essential for maintaining β-cell survival (Figure 1I,J). Altogether, our data demonstrate that FBXO28 expression correlates with β-cell survival and suggest FBXO28 as pro-survival protein in pancreatic β-cells.

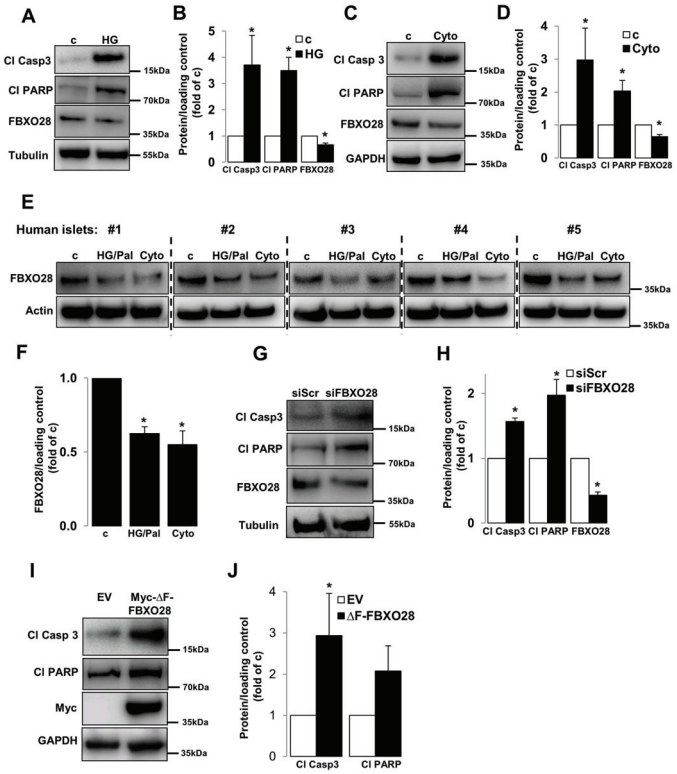

Figure 1. FBXO28 is reduced under diabetic conditions and its knockdown promotes β-cell apoptosis. INS-1E cells or isolated human islets were treated with (**A**,**B**) 22.2 mM glucose (HG), (**E**,**F**) the mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Pal), or (**C**–**F**) pro-inflammatory cytokines (2 ng/mL recombinant human IL-1β, and 1000 U/mL IFN-γ; cyto) or transfected with either control scrambled siRNA (siScr) or siRNA specific to FBXO28 (siFBXO28, **G**,**H**) or with either control empty vector (EV)- or Myc-conjugated ΔF-FBXO28-overexpressing plasmids (**I**,**J**) for 2 (INS-1E) or 3 (human islets) days. Representative Western blots of cleaved caspase-3 (Cl Casp3), cleaved PARP (Cl PARP) and FBXO28 protein levels (**A**,**C**,**E**,**G**,**I**) and pooled densitometric analyses from at least three independent experiments (INS-1E; **B**,**D**,**H**,**J**) or six human islet preparations (**F**) are shown. GAPDH or Tubulin or Actin was analyzed to ensure equal protein loading. Data show means ± SEM. * $p < 0.05$ compared to control conditions.

2.2. Overexpression of FBXO28 Protects β-Cells from Apoptosis

As loss of functional FBXO28 resulted in induction of β-cell apoptosis, we then investigated whether FBXO28 overexpression may restore β-cell survival under diabetogenic conditions. Myc-conjugated FBXO28 was overexpressed by liposome-mediated transfection of INS-1E cells (presented by immunoblotting of Myc; Figure 2) and then cultured under prolonged treatments with high glucose and inflammatory cytokines. Importantly, FBXO28 overexpression improved β-cell survival as indicated by diminished caspase-3 and PARP cleavage under both diabetogenic conditions (Figure 2A–D). Our data show that FBXO28 restoration acts as pro-survival signal to ameliorate the pro-diabetic milieu-induced β-cell apoptosis.

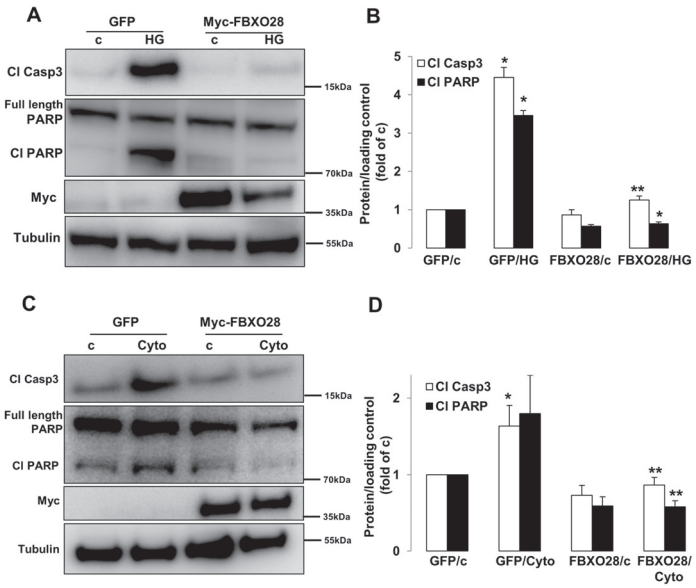

Figure 2. FBXO28 overexpression protects from β-cell apoptosis under diabetic conditions. INS-1E cells were transfected with either control GFP- or FBXO28-overexpressing plasmids and treated with (**A**) 22.2 mM glucose (HG) or (**C**) pro-inflammatory cytokines (2 ng/mL recombinant human IL-1β, and 1000 U/mL IFN-γ; cyto) for 2 days. Representative Western blots of cleaved caspase-3 (Cl Casp3), cleaved PARP (Cl PARP) and Myc protein levels (**A**,**C**) and pooled densitometric analyses from at least three independent experiments (**B**,**D**) are shown. Tubulin was analyzed to ensure equal protein loading. Data show means ± SEM. * $p < 0.05$ compared to GFP transfected control conditions, ** $p < 0.05$ compared to GFP transfected diabetic (HG/Cyto) conditions.

2.3. FBXO28 Does Not Regulate β-Cell Function

The major function of pancreatic β-cells is to control the production of insulin in response to fluctuations in blood glucose levels. To illustrate the potential impact of FBXO28 on the β-cell insulin secretory response and β-cell functional status, we performed glucose-stimulated insulin secretion (GSIS) as well as expression analyses of genes important for β-cell function, identity and maturation. Such essential experiments were performed in human islets isolated from control nondiabetic (Figure 3) and from organ donors with T2D (Figure 4). FBXO28 was overexpressed and silenced in isolated human islets by adenoviral systems. Successful overexpression and short hairpin RNA (shRNA)-mediated depletion of FBXO28 in human islets were assessed using RT-PCR-based analyses of FBXO28 mRNA levels as well as western blotting (Figure 3E,F,K,L) and immunofluorescence for GFP (Figure S1) in infected human islets. In contrast to the FBXO28 effect on β-cell survival, overexpression or knockdown did not significantly alter insulin content, GSIS and insulin stimulatory index in human islets (Figure 3). However, FBXO28 deficiency lowered the insulin stimulatory index by 1.5-fold, compared to infected control islets (Figure 3I), but because of variations among the different human islet isolations, these

data did not reach statistical significance ($p = 0.06$). To investigate whether FBXO28 changes modify expression levels of β-cell functional and maturation markers, we analyzed the expression of such genes including key β-cell transcription factors (*PDX1*, *NEUROD1*, *MAFA*, *NKX2.2*, and *NKX6.1*), the hormone insulin (*INS*), as well as important genes for glucose sensing and metabolism (*GCK* and *SLC2A2*) in human islets by RT-PCR (Figure 3D,J). Statistical analysis of pooled quantification data from 4 to 6 different human islet preparations indicated no significant differences for most of the analyzed genes upon FBXO28 knockdown or overexpression, respectively. Only *NEUROD1* highly correlated with FBXO28 expression levels; FBOXO28 overexpression induced *NEUROD1* (Figure 3D; $p < 0.05$), while *NEUROD1* was significantly reduced upon loss of FBXO28 in human islets (Figure 3J; $p < 0.05$), suggesting FBXO28 as previously uncharacterized regulator of *NEUROD1* expression.

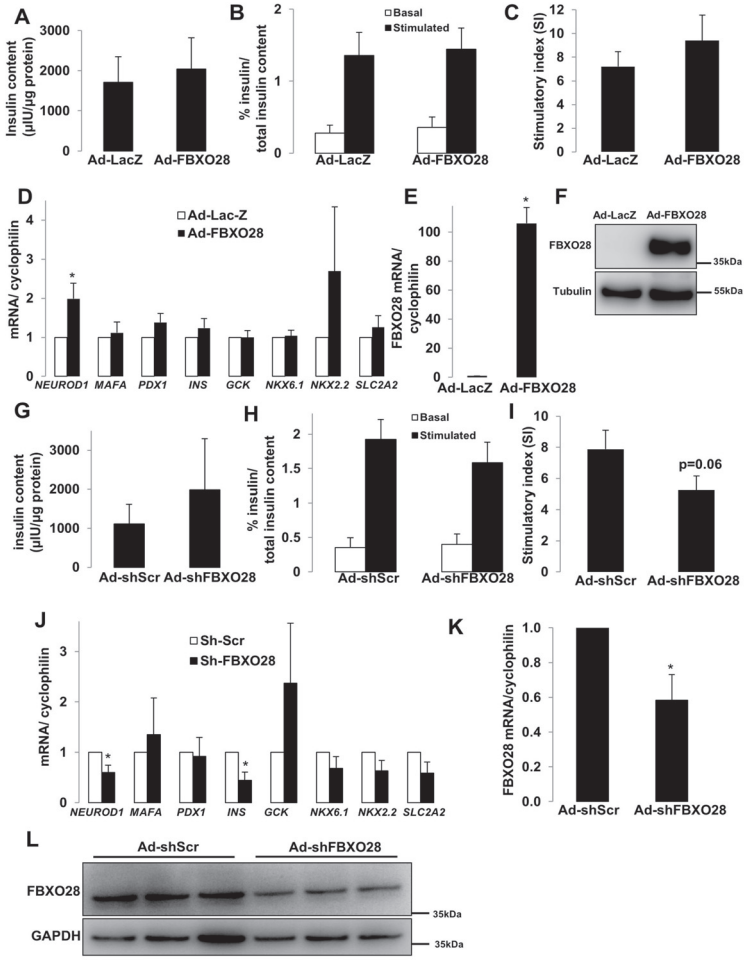

Figure 3. FBXO28 does not regulate β-cell function in human islets. Freshly isolated human islets of nondiabetic organ donors were infected with LacZ control or FBXO28 adenoviruses (**A**–**F**) or with Ad-GFP-shScr control or Ad-GFP-shFBXO28 (**G**–**L**) for 2 days. (**A**,**G**) Insulin content analyzed after GSIS and normalized to whole islet protein. (**B**,**H**) Insulin secretion during 1 h-incubation with 2.8 mM (basal) and 16.7 mM glucose (stimulated), normalized to insulin content. (**C**,**I**) The insulin stimulatory index denotes the ratio of secreted insulin during 1 h-incubation with 16.7 mM and 2.8 mM glucose. (**D**,**J**) RT-PCR for *NEUROD1, MAFA, PDX1, INS, GCK, NKX6.1, NKX2.2* and *SlC2A2* normalized to Cyclophilin. FBXO28 mRNA (**E**,**K**) and protein (**F**,**L**) expression in human islets confirm successful FBXO28 overexpression (**E**,**F**) and downregulation (**K**,**L**). Pooled data are from at least four independent experiments from at least four different human islet donors. Data show means ± SEM. * $p < 0.05$ compared to Ad-LacZ (**D**,**E**) or Ad-shScr (**J**,**K**).

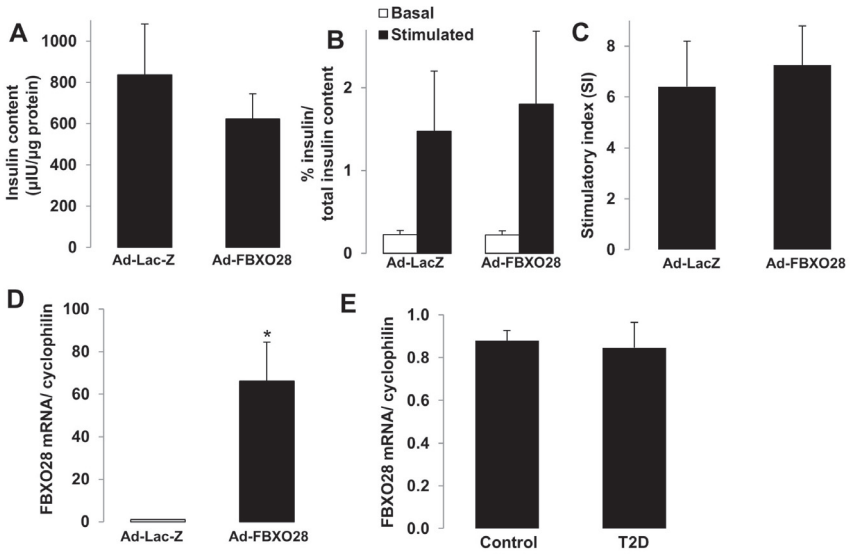

Figure 4. FBXO28 does not improve β-cell function in T2D human islets and *FBXO28* mRNA expression is unchanged in T2D islets. (**A**–**D**) Similar to non-diabetic human islets in Figure 3, freshly isolated human islets from patients with type 2 diabetes (T2D) were infected with LacZ control or FBXO28 adenoviruses for 1 day. (**A**) Insulin content analyzed after GSIS and normalized to whole islet protein; (**B**) Insulin secretion during 1 h-incubation with 2.8 mM (basal) and 16.7 mM glucose (stimulated), normalized to insulin content; (**C**) The insulin stimulatory index denotes the ratio of secreted insulin during 1 h-incubation with 16.7 mM and 2.8 mM glucose; (**D**) *FBXO28* mRNA expression in human T2D islets; (**E**) RT-PCR for *FBXO28* mRNA expression in human islets isolated from nondiabetic (n = 24) or individuals with T2D (n = 7), normalized to Cyclophilin. (**A**–**D**) Pooled data are from five independent experiments from five different human islet donors with confirmed T2D. Data show means ± SEM. * $p < 0.05$ compared to Ad-LacZ.

Despite the knowledge of NeuroD1 as known regulator of insulin gene expression required for β-cell maturation as well as important factor for β-cell survival [28–30], its modest up-regulation upon FBXO28 overexpression did not alter insulin mRNA or protein levels. This is not surprising as insulin gene expression is known to be regulated by a complex transcriptional network involving multiple transcription factors such as PDX1, NeuroD1 and MafA [30,31]. Thus, the up-regulation of one (*NEUROD1*) in the presence of two other unchanged critical transcription factors (*MAFA* and *PDX1*) does not seem to be sufficient to enhance insulin mRNA transcription. Nevertheless, *INS* mRNA levels significantly reduced together with *NEUROD1* upon loss of FBXO28 in human islets according to pooled data from four different human islet preparations (Figure 3J; $p < 0.05$). These data suggest that the mechanism of NeuroD1-dependent insulin gene transcription might be sensitive and operational to the loss of FBXO28, but that an increase of *NEUROD1* alone may not be sufficient to induce insulin production. Further mechanistic experiments are required to disclose such mechanism. Also, despite the tendency of lower expression of some genes including *SlC2A2* and *NKX2.2*, FBXO28 knockdown was not sufficient to significantly change the expression of other genes within the insulin machinery, besides *NEUROD1* and *INS* (Figure 3J), and also did not change insulin secretion itself (Figure 3H). This suggests that compensatory mechanisms for the FBXO28 loss are in place. Altogether, FBXO28 positively regulated *NEUROD1* mRNA expression, but does not have any significant effects on other tested genes, which is consistent with the lack of significant effects on intracellular insulin levels as well as on the insulin secretory response in human islets.

2.4. FBXO28 Overexpression Does Not Improve β-Cell Function in T2D Human Islets

The progressive defect in the insulin-secretory response of pancreatic β-cells is a key pathogenic hallmark of β-cell failure in T2D. As FBXO28 protein expression was reduced under diabetogenic conditions (Figure 1) and FBXO28 re-expression could restore β-cell survival (Figure 2), we then hypothesized that FBXO28 overexpression might restore β-cell function in already T2D diabetic islets in the ex vivo setting. Similarly to the experiments in Figure 3 performed in control islets from non-diabetic organ donors, FBXO28 was overexpressed by adenoviral infection of freshly isolated human islets from five different T2D organ donors (Figure 4). Insulin content and GSIS did not change significantly after overexpression of FBXO28 in human T2D islets (Figure 4A–D) indicating that FBXO28 down-regulation is dispensable for the defective insulin secretion in human T2D islets. This is in line with the unaltered insulin secretory function upon changes in FBXO28 expression in human islets from nondiabetic organ donors (Figure 3). Finally, we used our previously collected mRNA from isolated islets from age- and weight matched organ donors with T2D and respective nondiabetic controls for the *FBXO28* mRNA analysis. In contrast to the *FBXO28* reduction observed in islets from patients with T2D, compared to nondiabetic controls from microarray-based transcriptome analyses [16], we could not confirm such *FBXO28* RNA downregulation in T2D islets by classical RT-PCR (Figure 4E). In contrast to these ex vivo data is the downregulation of FBXO28 in β-cells and primary human islets under diabetogenic conditions on the protein level. It is therefore possible, that FBXO28, as substrate recognition partner of the SCF E3 ligase machinery, which mainly acts through its direct-direct protein interaction to recruit its targets, is rather regulated on the protein level, which is confirmed by the diabetogenic conditions in this study.

As a substrate-recruiting domain of the SCF-complex, FBXO28 plays a crucial role in recruiting proteins for degradation or localization in cellular processes such as cell cycle progression and cell proliferation. Recent research demonstrated that FBXO28 expression is regulated during the cell cycle through mechanism including CDK1/2-dependent stabilization [26]. In line with this, FBXO28 is required for proper mitotic progression and cell proliferation, through FBXO28-mediated non-proteolytic ubiquitination of MYC which regulates MYC-dependent transcription [26] and/or direct interaction with topoisomerase IIα, a key enzyme involved in fixing topological constraints of DNA [27]. Consistently, loss of FBXO28 impairs MYC-dependent transcription, hyper-proliferation and neoplastic growth and compromises mitotic progression [26,27]. Whether and to what extent FBXO28 is involved in cell cycle regulation, mitotic progression and proliferation of the hardly-dividing β-cell warrants further mechanistic investigations. By applying FBXO28 loss- and gain-of-function experiments, we show in the present study that: (i) FBXO28 protein levels were reduced in INS-1E β-cell as well as in isolated human islets under diabetic conditions; (ii) loss of FBXO28 as well as overexpression of its defective mutant (ΔF-FBXO28) induced basal β-cell apoptosis; and (iii) restoration of FBXO28 was sufficient to confer apoptotic resistance to β-cells under diabetic conditions; (iv) While FBXO28 regulated expression of β-cell transcription factor *NEUROD1* in isolated human islets, it did not alter β-cell function, or expression of several tested β-cell identity and functional genes; (v) Despite its effect on promoting β-cell survival, FBXO28 overexpression did not restore β-cell function in isolated islets from patients with T2D. All this shows that FBXO28 strongly regulated β-cell survival, whereas it did not have any significant independent effect on insulin secretion; neither in non-diabetic nor in T2D primary isolated human islets. This suggests a distinct cellular action of FBXO28 in β-cells. Further studies with the focus on the identification of β-cell specific targets of FBXO28, its underlying mechanism of action as well as thorough investigations of β-cell-specific FBXO28 transgene/knockout mice are under way and will hopefully greatly advance our current understanding of the physiological regulation and function of FBXO28 at the cellular, molecular and organismic level in the control of metabolism.

3. Materials and Methods

3.1. Islet Isolation, Cell Culture, and Treatment

Human islets were isolated from pancreases of non-diabetic organ donors as well as from individuals with T2D at Lille University and at ProdoLabs and cultured on extracellular matrix (ECM) coated dishes as described formerly [32]. The clonal rodent β-cell line INS-1E was provided by Dr. Claes Wollheim, Geneva and Lund University. Human islets were cultured in complete CMRL-1066 (Invitrogen, Bleiswijk, Netherlands) medium at 5.5 mM glucose and INS-1E cells in complete RPMI-1640 (Invitrogen) medium at 11.1 mM glucose. INS-1E cells and isolated human islets were treated with complex diabetogenic conditions as described previously [3]. Ethical approval for the utilization of islets was granted by the Ethics Committee of the University of Bremen.

3.2. Transfections

FBXO28 and ΔF-FBXO28 plasmids [26] (kindly provided by Olle Sangfelt, Karolinska Institute, Stockholm, Sweden) and ON-TARGETplus SMARTpool technology (mix of 100 nM siRNAs directed against rat FBXO28; Dharmacon, Lafayette, CO, USA) were used to overexpress FBXO28/silence FBXO28 in INS-1E cells as described before [3].

3.3. Adenoviral Infection

The adenoviruses Ad-h-FBXO28 expressing human FBXO28 and Ad-GFP-U6-hFBXO28-shRNA expressing GFP and human FBXO28 shRNA were obtained from Vector Biolabs (Malvern, PA, USA). Ad-LacZ or Ad-GFP-U6-shRNA were used as respective controls. For transduction, human islets were plated on ECM dishes for 24 h; islets were infected with adenoviruses at multiplicity of infection (MOI) of 100 in FCS-free CMRL-1066 medium. After 4 h incubation, human islets were washed and incubated with fresh complete media. GSIS, RNA and protein extractions were performed 24 or 48 h after infection.

3.4. Western Blot Analysis

Western blotting was performed as depicted previously [3]. After the treatment periods, INS-1E cells or human islets were washed twice with ice-cold PBS and lysed with RIPA lysis buffer containing protease and phosphatase Inhibitors (Pierce, Rockford, IL, USA). Protein concentrations were measured by the BCA protein assay (Pierce). Lysates were fractionated by NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electrically transferred onto PVDF membranes. Membranes were blocked in 2.5% non-fat dry milk (Cell Signaling Technology, Danvers, MA, USA) and 2.5% BSA (Sigma, St. Louis, MO, USA) for 1 h at room temperature and incubated overnight at 4 °C with rabbit anti-cleaved caspase-3 (#9664), rabbit anti-PARP (#9532), rabbit anti-cleaved PARP (rat specific #9545), mouse anti-Myc (#2276), rabbit anti-tubulin (#2146), rabbit anti-GAPDH (#2118) and rabbit anti-β-actin (#4967) (all Cell Signaling Technology), and rabbit anti-FBXO28 (#ab154068) (Abcam, Cambridge, UK) followed by horseradish-peroxidase-linked anti-rabbit IgG (Jackson, West Grove, PA, USA). Membranes were developed by a chemiluminescence assay system (Pierce) and evaluated with DocIT®LS image acquisition 6.6a (UVP Bio Imaging Systems, Upland, CA, USA).

3.5. Glucose-Stimulated Insulin Secretion

Glucose-stimulated insulin secretion (GSIS) was performed in human islets as described previously [3]. Briefly islets were pre-incubated in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose and 0.5% BSA. KRB was then replaced by KRB 2.8 mM glucose for 1 h (basal), followed by an additional 1 h in KRB 16.7 mM glucose (stimulated). Total protein content was extracted with RIPA buffer and protein concentration determined by the BCA assay (Pierce). Insulin levels

were measured using human insulin ELISA (ALPCO Diagnostics, Salem, NH, USA). Secreted and intracellular insulin was normalized to total insulin and total protein content.

3.6. RNA Extraction and RT-PCR Analysis

RNA was isolated from cultured human islets by utilizing Trizol extraction method (TriFast-PEQLAB Biotechnology, Erlangen, Germany), and cDNA synthesis and quantitative RT-PCR was performed as previously described [3]. For evaluation, we used the Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with TaqMan(R) Fast Universal PCR Master Mix for TaqMan gene expression assays (Applied Biosystems) for *PDX1* (Hs00236380_m1), *SLC2A2* (Hs01096905_m1), *GCK* (Hs01564555_m1), *INS* (Hs02741908_m1), *NKX2.2* (Hs00159616_m1), *MAFA* (Hs01651425_s1), *NKX6.1* (Hs00232555_m1), *NEUROD1* (Hs01922995_s1), *FBXO28* (Hs00429691_m1), and *PPIA* (Hs99999904_m1).

3.7. Statistical Analysis

All values were expressed as means ± SEM and p value < 0.05 analyzed by unpaired student t-test for comparison of two groups was considered statistically significant.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/4/975/s1.

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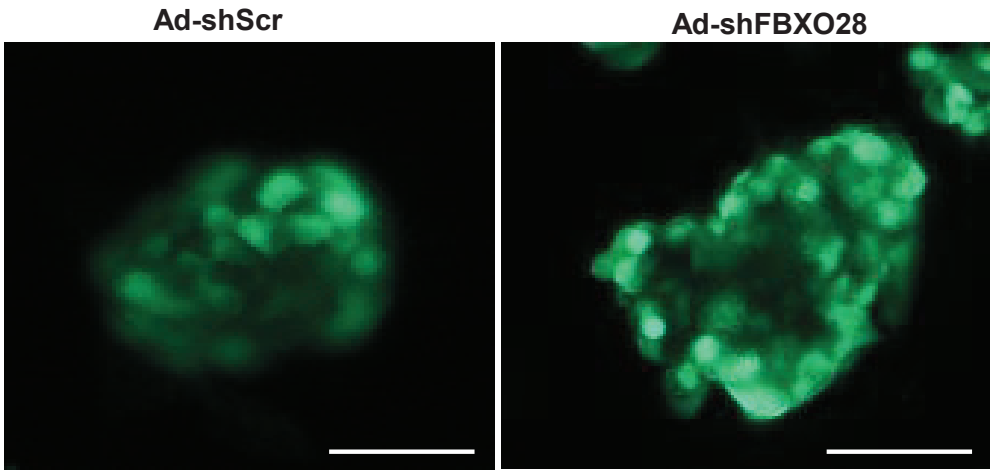

Supplementary Figure 1. Representative immunofluorescence of GFP in infected human islets with Ad-GFP-shScr control or Ad-GFP-shFBXO28. Scale bar represents 50μm.

Manuscript 2

2.2. Loss of deubiquitinase USP1 blocks pancreatic β-cell apoptosis by inhibiting DNA damage response

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Designed, performed and analyzed all the experiments, and wrote the paper. BL and KA assisted me in performing and analyzing Figure 6G-I and Figure 7.

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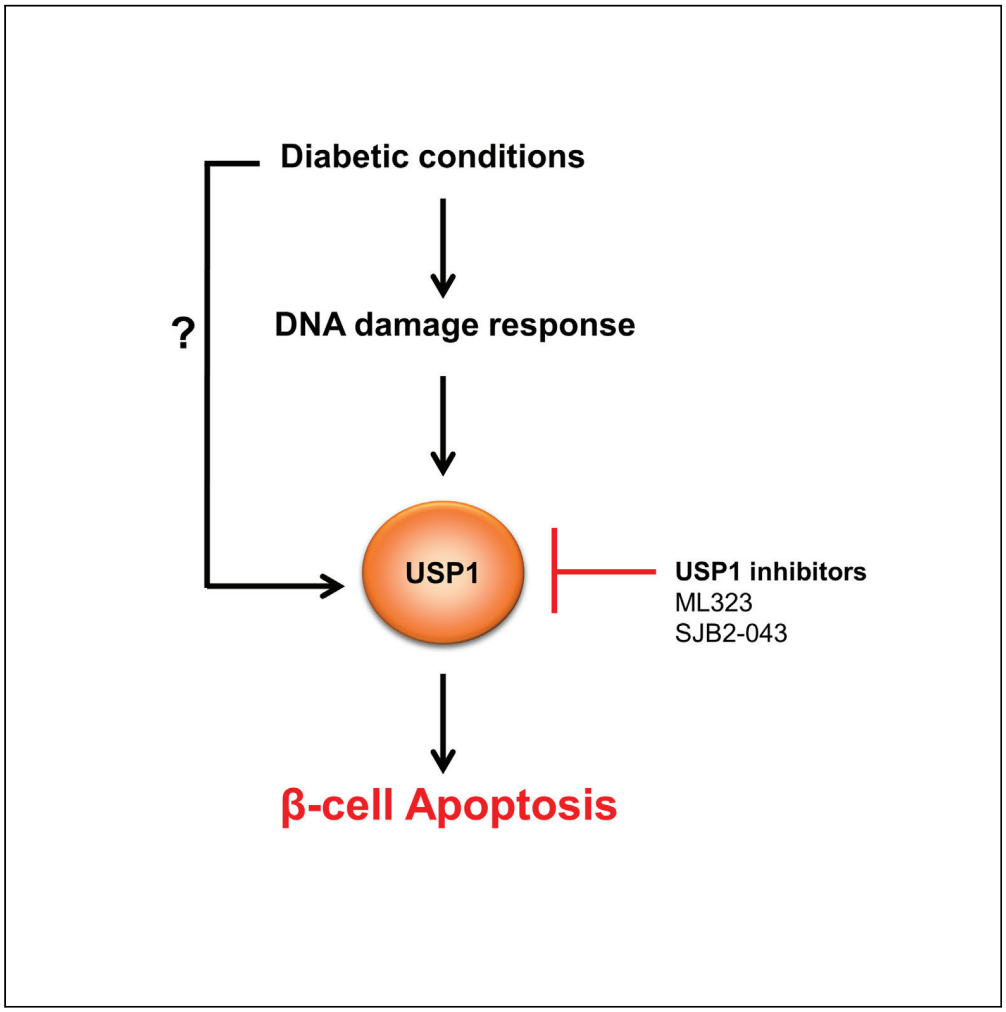

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HIGHLIGHTS

Genetic and chemical inhibition of USP1 promoted β-cell survival

USP1 inhibitors blocked β-cell death in human islets without affecting β-cell function

USP1 inhibition reduced DDR signals in stressed β-cells

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Loss of Deubiquitinase USP1 Blocks Pancreatic β-Cell Apoptosis by Inhibiting DNA Damage Response

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SUMMARY

Impaired pancreatic β-cell survival contributes to the reduced β-cell mass in diabetes, but underlying regulatory mechanisms and key players in this process remain incompletely understood. Here, we identified the deubiquitinase ubiquitin-specific protease 1 (USP1) as an important player in the regulation of β-cell apoptosis under diabetic conditions. Genetic silencing and pharmacological suppression of USP1 blocked β-cell death in several experimental models of diabetes *in vitro* and *ex vivo* without compromising insulin content and secretion and without impairing β-cell maturation/identity genes in human islets. Our further analyses showed that USP1 inhibition attenuated DNA damage response (DDR) signals, which were highly elevated in diabetic β-cells, suggesting a USP1-dependent regulation of DDR in stressed β-cells. Our findings highlight a novel function of USP1 in the control of β-cell survival, and its inhibition may have a potential therapeutic relevance for the suppression of β-cell death in diabetes.

INTRODUCTION

Loss of function and/or mass of insulin-producing pancreatic β-cells is a hallmark of both type 1 and 2 diabetes (T1D/T2D) (Butler et al., 2003; Kurrer et al., 1997; Mathis et al., 2001; Rhodes, 2005; Vetere et al., 2014). Pancreatic β-cell death is a critical pathogenic factor contributing to the declined β-cell mass in both T1D and T2D (Ardestani et al., 2014; Butler et al., 2003; Kurrer et al., 1997; Marselli et al., 2014; Masini et al., 2009; Mathis et al., 2001; Meier et al., 2005; Rahier et al., 2008; Rhodes, 2005; Tomita, 2010). Also, β-cell dedifferentiation (Cinti et al., 2016; Jeffery and Harries, 2016; Talchai et al., 2012) and impaired adaptive proliferation (Ardestani and Maedler, 2018; Tiwari et al., 2016) have been proposed as potential additional causes for this diminished β-cell mass in diabetes. Although immune-cell-mediated events predominate in T1D (Chatenoud, 2010), metabolic factors such as elevated levels of glucose, fatty acids, and islet amyloid polypeptide (IAPP), alongside with pro-inflammatory cytokines, drive β-cell loss and dysfunction in T2D (Alejandro et al., 2015; Ardestani et al., 2018; Donath et al., 2013; Haataja et al., 2008; Huang et al., 2007; Maedler et al., 2002; Poitout and Robertson, 2008; Robertson et al., 2004; Yuan et al., 2017). Excessive β-cell death is commonly seen in the islets of both patients with T1D and lean and obese patients with T2D as determined by multiple complementary approaches (Butler et al., 2003; Masini et al., 2009; Meier et al., 2005; Tomita, 2010). Counter-intuitively, higher levels of β-cell apoptosis in autopsy pancreases from patients with established diabetes inversely correlate with the insulin-positive β-cell area. But even at a pre-diabetic stage, i.e., in at-risk individuals who progressed to T1D, β-cell death is evident and accompanied with diminished insulin secretion (Herold et al., 2015). Correspondingly, in patients with impaired glucose tolerance before T2D diagnosis, loss of β-cells is apparent and correlates with elevated fasting glucose levels (Ritzel et al., 2006); this gradually progresses when hyperglycemia is established and β-cells are unable to sustain insulin production under a higher metabolic demand. Indeed, pancreatic β-cells show a relatively higher susceptibility to apoptosis together with lower stress-induced protective responses compared with many other cell types, including the neighboring pancreatic α-cells. For instance, a recent proteomic analysis shows a very weak induction of β-cell's reactive oxygen species (ROS)-detoxifying enzymes in response to inflammatory assault (Gorasia et al., 2015). This confirms previous observations showing very low levels of protective anti-oxidative enzymes in β-cells (Grankvist et al., 1981; Lenzen, 2008; Lenzen et al., 1996; Tiedge et al., 1997). Consistently, human β-cells are much more sensitive to apoptosis than α-cells in response to T2D-related metabolic stressors (Marroqui et al., 2015). All these seem to represent an "Achilles heel" through which

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diabetogenic stimuli trigger rapid β-cell death and accelerate the collapse in response to environmental stress and demand.

The ubiquitin-proteasome system (UPS) is a highly regulated key intracellular protein degradation pathway, which consists of an enzymatic cascade controlling protein ubiquitination and has important functions in several essential biological processes, such as cell survival, proliferation, development, and DNA damage response (DDR) (Schmidt and Finley, 2014). The UPS is a process of post-translational modification of targeted protein by covalent attachment of one or more ubiquitins to lysine residues by an *E3* ubiquitin ligase. This is antagonized by enzyme deubiquitinases (DUBs), such as ubiquitin-specific proteases (USPs). The UPS is primarily responsible for the degradation and clearance of misfolded or damaged proteins as well as of dysfunctional organelles, which compromise cellular homeostasis. Abnormalities in the UPS machinery have been linked to the pathogenesis of many diseases, including cancer, immunological and neurological disorders (Frescas and Pagano, 2008; Schmidt and Finley, 2014; Zheng et al., 2016), as well as β-cell failure in diabetes (Broca et al., 2014; Bugliani et al., 2013; Costes et al., 2011, 2014; Hartley et al., 2009; Hofmeister-Brix et al., 2013; Kaniuk et al., 2007; Litwak et al., 2015). A member of the USP family, ubiquitin-specific protease 1 (USP1), is one of the best known DUBs responsible for removing ubiquitin from target proteins and thus influences several cellular processes such as survival, differentiation, immunity and DDR (Garcia-Santisteban et al., 2013; Liang et al., 2014; Yu et al., 2017). Although USP1 was initially identified as a novel component of the Fanconi anemia DNA repair pathway (Nijman et al., 2005), extensive subsequent studies revealed a pleotropic function of USP1 and identified novel interacting partners and signaling for USP1 action and regulation in normal physiological conditions and in disease states such as tumorigenesis (Garcia-Santisteban et al., 2013; Liang et al., 2014; Yu et al., 2017). An array-based assay identified reduced USP1 mRNA expression in islets from patients with T2D (Bugliani et al., 2013). As the consequent effects of USP1 in diabetes and especially in the pancreatic β-cell were completely unknown so far, we investigated the role and the mechanism of action of USP1 on β-cell survival under diabetic conditions using clonal β-cells and isolated primary human islets. Although USP1 protein expression was unchanged in a diabetic milieu, we identified a robust protective effect on β-cell survival by USP1 inhibition.

RESULTS

USP1 Knockdown Protects β-cells from Apoptosis Under Diabetic Conditions

Transcriptome analysis of islets isolated from healthy individuals as well as from patients with T2D showed consistent alteration of genes of UPS components, including members of the USP family such as USP1 (Bugliani et al., 2013). Because USP1 is involved in signaling pathways associated with DDR and survival (Liang et al., 2014), we aimed here to identify whether USP1 regulates apoptosis in β-cells under diabetogenic conditions. USP1 was expressed in protein lysates extracted from both human and mouse islets (data not shown) and INS-1E cells (Figure 1). The total protein level was not significantly changed in response to a pro-diabetic milieu in INS-1E cells (Figure 1). To evaluate the function of USP1 in the regulation of β-cell survival, USP1 was depleted in rat INS-1E β-cells by transfection with siUSP1 (Figure S1) and thereafter cultured long term with high glucose concentrations (glucotoxicity; Figures 1A and 1B), a combination of high glucose with saturated free fatty acid palmitate (glucolipotoxicity; Figures 1C and 1D), and a cocktail of pro-inflammatory cytokines (interleukin-1 beta [IL-1β], interferon gamma [IFN-γ], and tumor necrosis factor alpha [TNF-α]; Figures 1E and 1F). Consistent with our previous observations, long-term culture with elevated glucose, glucose/palmitate, and cytokines robustly induced β-cell apoptosis (Ardestani et al., 2014; Yuan et al., 2016a, 2016b). Knockdown of USP1 markedly reduced the levels of glucose-, glucose/palmitate-, and cytokine-induced apoptosis as indicated by decreased levels of hallmarks of apoptosis, namely, caspase-3 and its downstream target poly(ADP-ribose) polymerase (PARP) cleavage (Figures 1A–1F). These data indicate that loss of USP1 confers apoptosis resistance to β-cells against stress-induced cell death.

Small Molecule USP1 Inhibitors Block β-Cell Apoptosis Under Diabetic Conditions

Several USP1 small molecule inhibitors have been developed recently. Quantitative high-throughput screen and subsequent medicinal chemistry identified compound ML323 (Figure 2A) as a highly potent selective inhibitor of USP1 with excellent specificity, when compared with other DUBs, deSUMOylase, deneddylase, and unrelated proteases (Dexheimer et al., 2010; Liang et al., 2014). ML323 was able to potently inhibit USP1 activity in a dose-dependent manner in several complementary *in vitro* assays as well as in cellular models as represented by increased monoubiquitination of USP1 known substrates proliferating cell nuclear antigen (PCNA) and Fanconi anemia group D2 protein (FANCD2) (Dexheimer et al., 2010; Liang

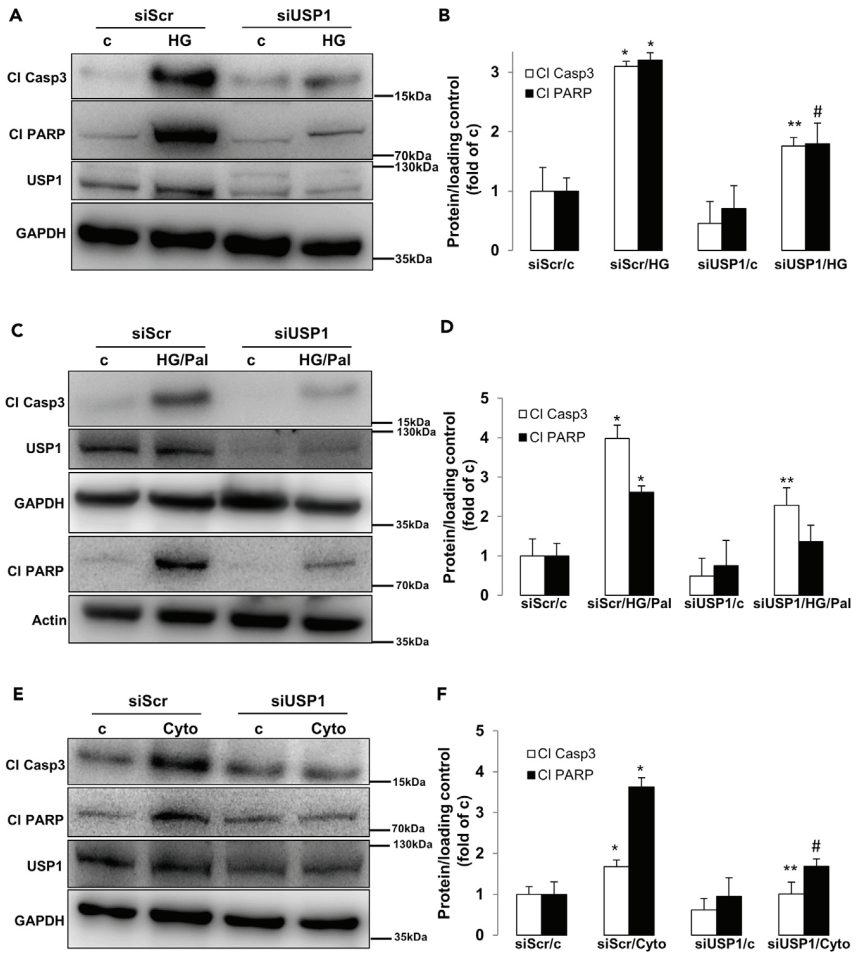

Figure 1. USP1 Knockdown Protects β-Cell from Apoptosis Under Diabetic Conditions

(A–F) INS-1E cells were seeded at 300,000 cells/well and transfected with either control scrambled siRNA (siScr) or siRNA specific to USP1 (siUSP1) and treated with (A and B) 22.2 mM glucose (HG), (C and D) a mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Pal), or (E and F) pro-inflammatory cytokines (2 ng/mL recombinant human IL-1β, 1000 U/mL TNF-α, and 1000 U/mL IFN-γ; Cyto) for 2 days. Representative Western blots (A, C, and E) and quantitative densitometry analysis (B, D, and F) of cleaved caspase 3 (Cl Casp3) and cleaved PARP (Cl PARP) protein levels are shown. Data are pooled from at least three independent cell line experiments. Data show means ±SEM. *$p < 0.05$ siScr treated compared with siScr control conditions. **$p < 0.05$ siUSP1-treated compared with siScr-treated conditions. #$p = 0.05$ compared with HG (B) or Cyto (F). See also Figure S1 for USP1 quantification.

et al., 2014). Inhibition of USP1 was also confirmed in INS-1E cells treated with ML323 as indicated by the accumulation of ubiquitinated PCNA, a USP1 downstream substrate (Figures S2A and S2B). To confirm the anti-apoptotic action of USP1 loss obtained by the genetic approach, we used ML323 to block USP1 activity in INS-1E cells exposed to diabetic conditions. USP1 inhibition by ML323 at two concentrations (5 and 10 μM), which proved to be non-toxic at basal levels (data not shown), potently inhibited the induction of caspase-3 and PARP cleavage triggered by elevated glucose (Figures 2B and 2C), glucose/palmitate (Figures 2D and 2E), and cytokines (Figures 2F and 2G), further demonstrating the pro-apoptotic activity of USP1 in the presence of the diabetic milieu. Also, by using an ubiquitin-rhodamine-based high-throughput screening, Mistry et al. identified SJB2-043 (Figure 3A) as another potent selective small-molecule inhibitor of USP1. SJB2-043 blocked the deubiquitinating enzyme activity of USP1 *in vitro* with an IC50 in the nanomolar range (Mistry et al., 2013). To further confirm the utility of USP1 blockade in protecting β-cells from apoptosis, we tested the effect of SJB2-043 on β-cell survival in the presence of diabetogenic conditions. Consistent with our genetic and pharmacological approaches using siRNA against USP1 and the USP1 inhibitor ML323, USP1 inhibition by SJB2-043 at two concentrations (20 and 50 nM) attenuated

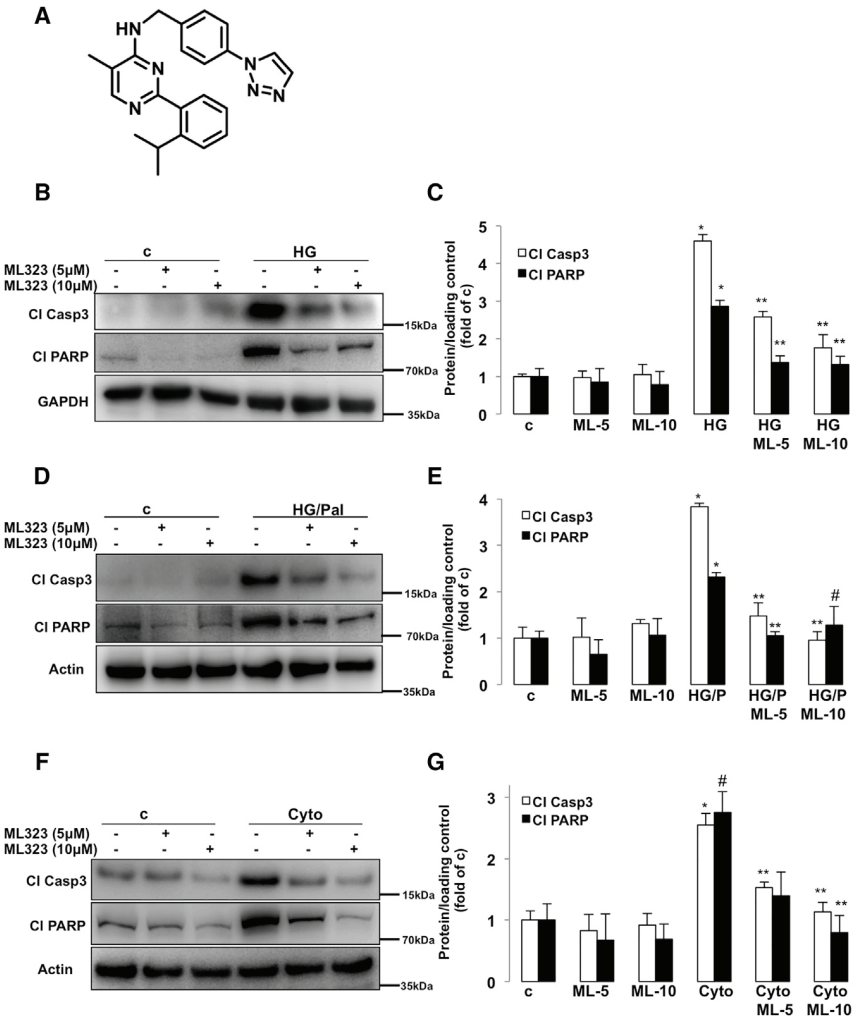

Figure 2. USP1 Inhibitor ML323 Blocks β-Cell Apoptosis Under Diabetic Conditions

(A) Chemical structure of ML323.

(B–G) About 500,000 INS-1E cells/well treated with or without USP1 inhibitor ML323 were exposed to 22.2 mM glucose (HG; B and C), a mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Pal; D and E), or pro-inflammatory cytokines (2 ng/mL recombinant human IL-1β, 1000 U/mL TNF-α, and 1000 U/mL IFN-γ; Cyto; F and G) for 2 days. Representative Western blots and quantitative densitometry analysis of cleaved caspase 3 (Cl Casp3) and cleaved PARP (Cl PARP) protein levels are shown. Data are pooled from at least three independent cell line experiments. Data show means ±SEM.

*$p < 0.05$ treated compared with control conditions. **$p < 0.05$ inhibitor-treated compared with treated conditions.

#$p = 0.05$ compared with HG/P (E) or untreated control (G) alone. See also Figure S2 for PCNA ubiquitination.

β-cell apoptosis as represented by the robust reduction of cleaved caspase-3 and PARP induced by glucotoxicity (Figures 3B and 3C), glucolipotoxicity (Figures 3D and 3E), and pro-inflammatory cytokines (Figures 3F and 3G). Altogether, small molecule inhibitors of USP1 improved β-cell survival under diabetic conditions and recapitulated the β-cell protective effect of USP1 silencing.

USP1 Inhibition Protects Human Islets from Apoptosis without Compromising Their Insulin Secretory Function

To test the efficacy of USP1 inhibitors in blocking β-cell apoptosis in human islets with physiologically more relevant properties for human disease, human islets isolated from nondiabetic organ donors were treated with USP1 inhibitors ML323 and SJB2-043 and then exposed to the diabetogenic milieu of glucolipotoxicity and pro-inflammatory cytokines IL-1β and IFN-γ. Caspase-3 cleavage triggered by high glucose/palmitate as well as by the mixture of cytokines was counteracted by ML323 in isolated human islets, confirmed

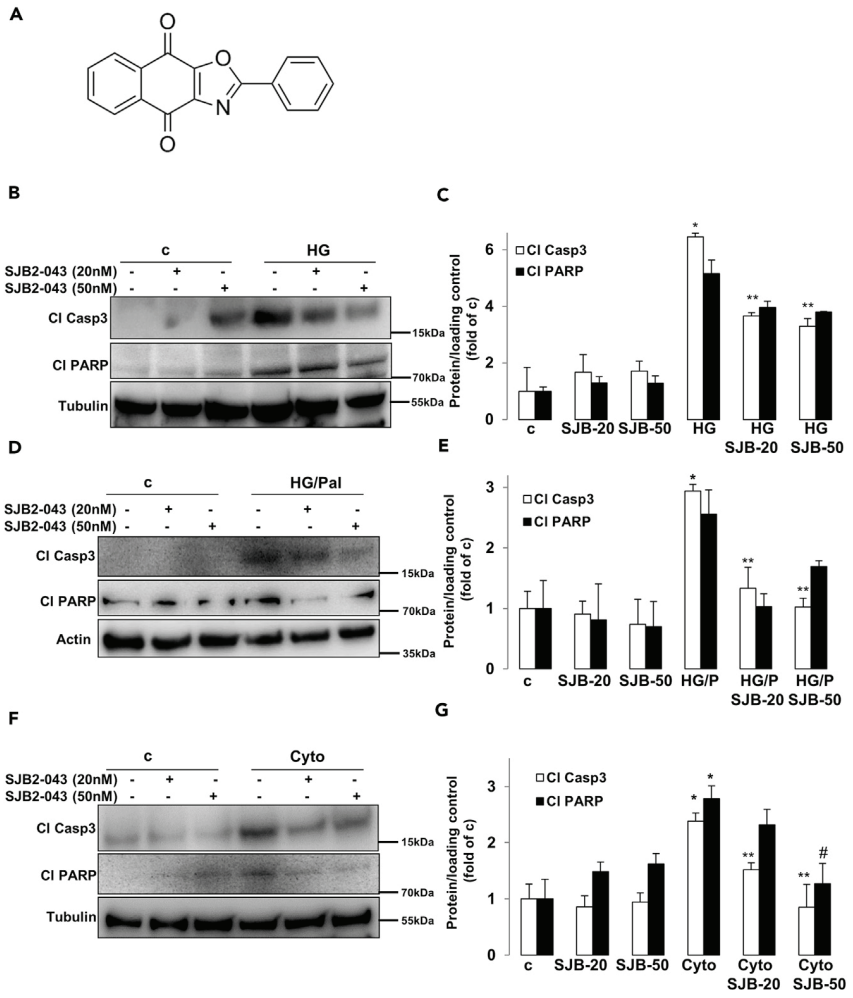

Figure 3. USP1 Inhibitor SJB2-043 Blocks β-Cell Apoptosis Under Diabetic Conditions

(A) Chemical structure of SJB2-043.

(B–G) About 500,000 INS-1E cells/well treated with or without USP1 inhibitor SJB2-043 were exposed to 22.2 mM glucose (HG; B and C), a mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Pal; D and E), or pro-inflammatory cytokines (2 ng/mL recombinant human IL-1β, 1000 U/mL TNF-α, and 1000 U/mL IFN-γ; Cyto; F and G) for 2 days. Representative Western blots and quantitative densitometry analysis of cleaved caspase 3 (Cl Casp3) and cleaved PARP (Cl PARP) protein levels are shown. Data are pooled from at least three independent cell line experiments except for cl PARP in C (n = 2). Data show means ±SEM. *$p < 0.05$ treated compared with control conditions. **$p < 0.05$ inhibitor-treated compared with treated conditions. #$p = 0.05$ compared with Cyto (G) alone.

independently by four batches of human islet preparations (Figures 4A–4D). Similar data were obtained using SJB2-043 as approach to target USP1 in isolated human islets (Figures 4E–4H). To confirm protection from apoptosis, *in situ* TUNEL together with insulin double-staining was performed to validate our Western blot data on the level of β-cells. Again, both USP1 inhibitors ML323 and SJB2-043 fully protected human islet β-cells from apoptosis induced by prolonged exposure to elevated glucose/palmitate and to the mixture of cytokines (Figures 4I and 4J), confirming the promising efficacy of USP1 inhibitors in rescuing human β-cells from apoptosis. Although strategies to interfere with the intracellular cell death mechanisms to halt β-cell failure in diabetes are highly promising, they should not compromise the insulin secretory function of pancreatic β-cells. As previously reported, modulation of key endogenous anti-apoptotic proteins in β-cells impairs β-cell function (Luciani et al., 2013). Therefore, we sought to determine whether the chemical blockade of USP1 has any impact on insulin secretion and expression of key β-cell maturation and identity genes. Therefore, we probed the influence of USP1 inhibition on the ability of human islets to secrete insulin in response to glucose. Human islets treated with ML323 and SJB2-043 showed no alteration

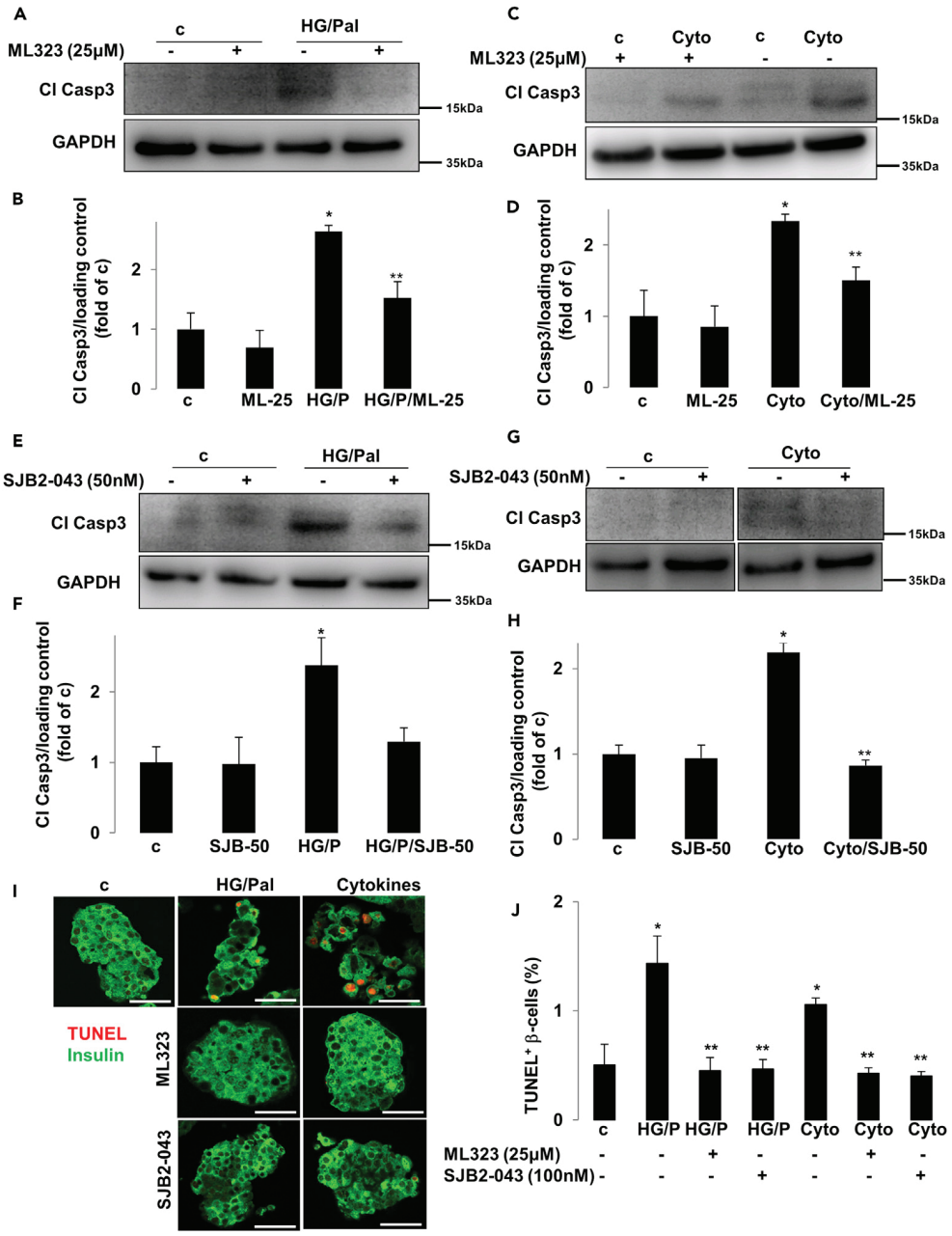

Figure 4. Inhibition of USP1 Promotes β-Cell Survival in Isolated Human Islets

(A–J) Isolated human islets (150–200 islets/dish) treated with or without USP1 inhibitors ML323 or SJB2-043 were exposed to a mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Pal) (A, B, E, F, I, and J) or to pro-inflammatory cytokines (2 ng/mL recombinant human IL-1β and 1000 U/mL IFN-γ; Cyto; C, D, G–J) for 2 days. Representative Western blots and quantitative densitometry analysis of cleaved caspase 3 (Cl Casp3) protein levels are shown. (G) All lanes are from the same gel but were run noncontiguously. (I and J) Human pancreatic islets were fixed, paraffin embedded, and stained for the TUNEL assay and insulin. Representative images (I) and quantitative percentage of TUNEL-positive β-cells (J) are shown. Scale bars represent 50 μm. Data are pooled from at least three human islet preparations. Data show means ±SEM. *$p < 0.05$ stimuli treated compared with control conditions. **$p < 0.05$ inhibitor-treated compared with treated conditions.

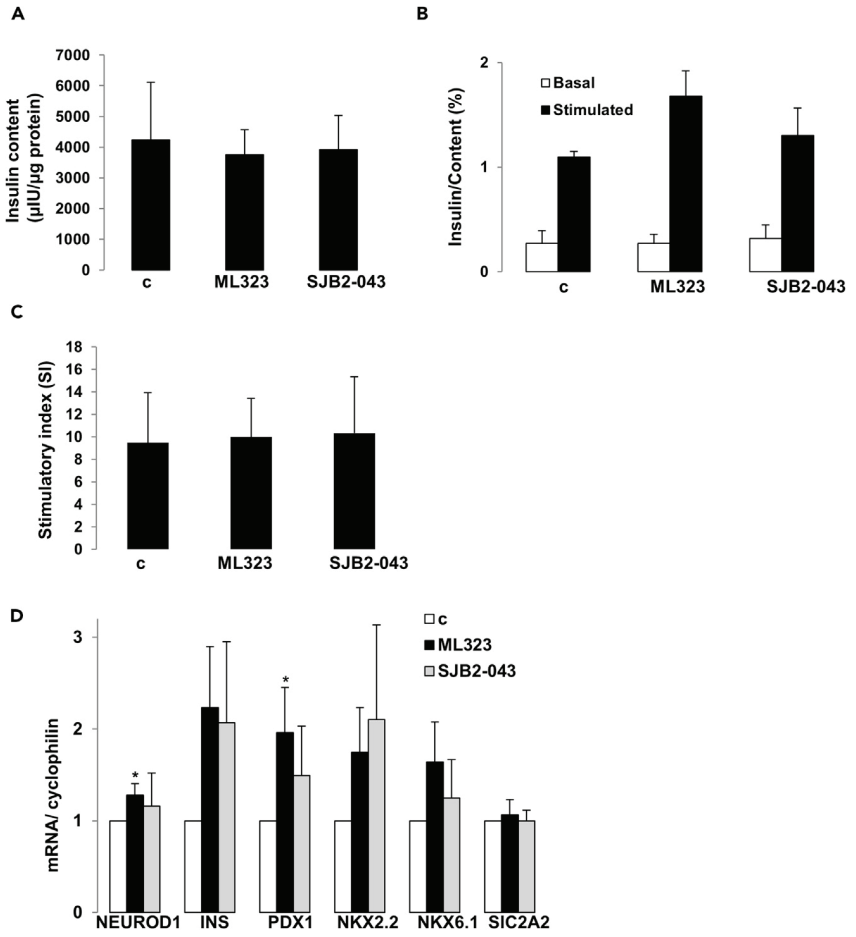

Figure 5. Impact of USP1 Inhibition on Insulin Content, Secretion, and Expression of Functional β-Cell Genes in Isolated Human Islets

(A–D) Isolated human islets were treated with or without USP1 inhibitors ML323 or SJB2-043 for 2 days (30 islets/dish for A–C; 150–200 islets/dish for D). (A) Insulin content analyzed after GSIS and normalized to whole islet protein. (B) Insulin secretion during 1-hr incubation with 2.8 (basal) and 16.7 mM glucose (stimulated), normalized to insulin content. (C) The insulin stimulatory index denotes the ratio of secreted insulin during 1-hr incubation with 16.7 and 2.8 mM glucose. (D) RT-PCR for *NEUROD1*, *INS*, *PDX1*, *NKX2.2*, *NKX6.1*, and *SIC2A2*, normalized to cyclophilin. Tubulin normalization delivered similar results. Pooled data are from four independent experiments from four different human islet donors. Data show means ±SEM. *$p < 0.05$ compared with control conditions.

in the intracellular insulin content (Figure 5A), and glucose-stimulate insulin secretion (GSIS) was maintained (Figures 5B and 5C), indicating that USP1 inhibition does not affect the human islet insulin secretory function. To determine whether USP1 inhibitors caused a loss of β-cell identity or de-differentiation, expression of functional genes, including insulin (*INS*), key β-cell transcription factors (*PDX1*, *NEUROD1*, *NKX2.2*, and *NKX6.1*), as well as the critical β-cell glucose transporter (*SIC2A2*), were measured by reverse transcription polymerase chain reaction (RT-PCR) in human islets. Our data showed that not only all genes were preserved in USP1 inhibitors-treated human islets cells but also *NEUROD1* and *PDX1* were significantly increased by ML323 (Figure 5D), suggesting full preservation of β-cell function and functional identity genes upon inhibition of USP1 in human islets. Our data show that inhibition of USP1 efficiently blocked human β-cell apoptosis without compromising function.

USP1 Inhibition Suppressed the DNA Damage Response in Pancreatic β-cells

Complex intracellular networks, namely, the DDR, monitor genome integrity and stability by sensing DNA damage and controlling DNA repair pathways and damage tolerance processes (Polo and Jackson, 2011). In principle, if the level of DDR is greater than the cellular capacity, programmed cell death is activated to

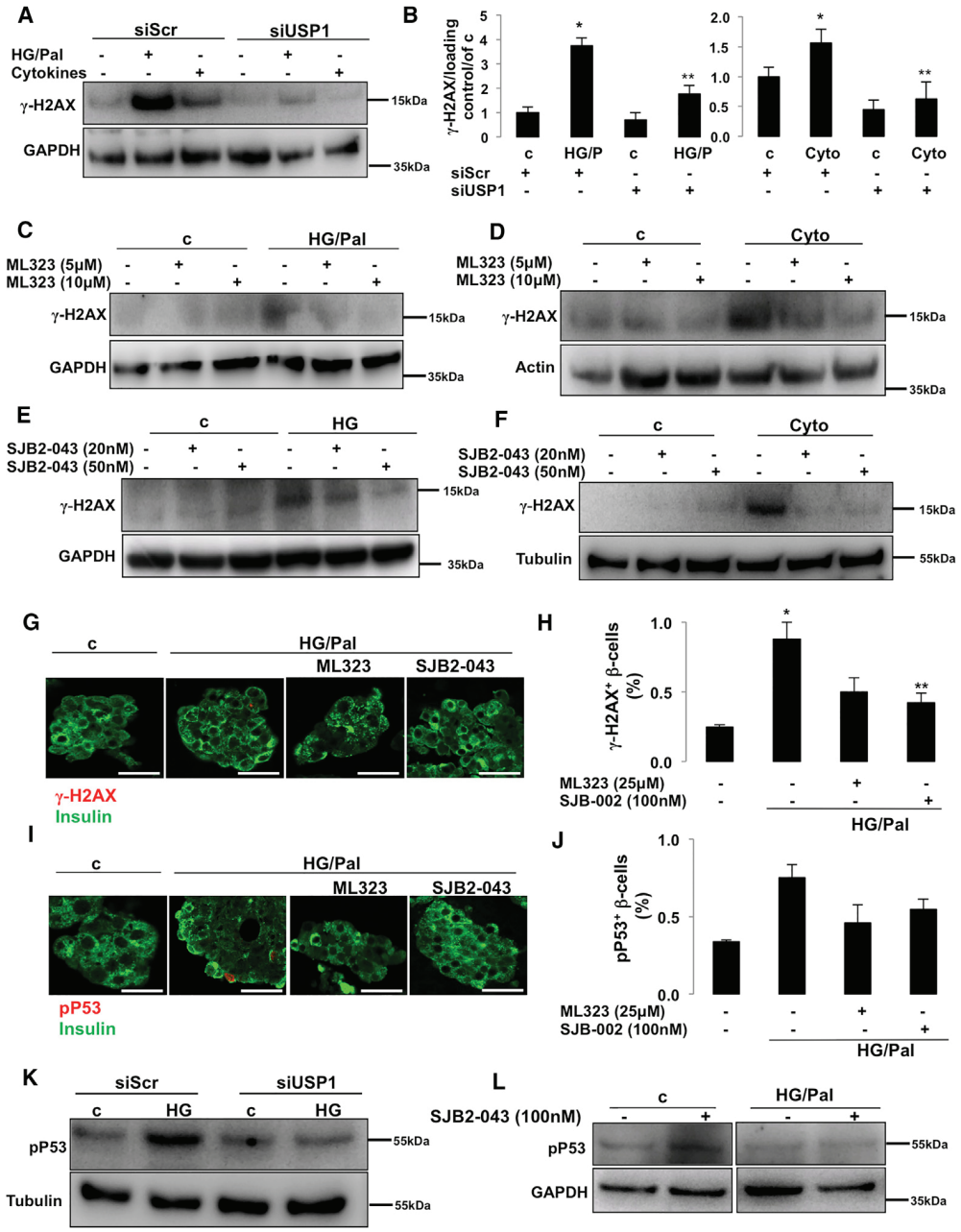

Figure 6. Inhibition of USP1-Suppressed DDR

(A, B, and K) INS-1E cells seeded at 300,000 cells/well were transfected with either control siScr or siUSP1 and treated with a mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Pal) or pro-inflammatory cytokines (2 ng/mL recombinant human IL-1β, 1000 U/mL TNF-α, and 1000 U/mL IFN-γ; Cyto). Representative Western blot (A and K) and quantitative densitometry analysis (B) of γ-H2AX and p-p53 proteins are shown.

(C–F) About 500,000 INS-1E cells/well treated with or without USP1 inhibitors ML323 (C and D) or SJB2-043 (E and F) were exposed to a mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Pal; C), 22.2 mM glucose (HG; E), or pro-inflammatory cytokines (2 ng/mL recombinant human IL-1β, 1000 U/mL TNF-α, and 1000 U/mL IFN-γ; Cyto; D and F) for 2 days. Data are pooled from at least three independent cell line experiments. Tubulin loading control in Figure 5F is re-used from the same experiment shown in Figure 3F.

(G–J and L) Isolated human islets (150–200 islets/dish) treated with or without USP1 inhibitors ML323 or SJB2-043 were exposed to a mixture of 22.2 mM glucose and 0.5 mM palmitate (HG/Pal) for 2 days. (G–J) Human pancreatic islets were fixed, paraffin embedded, and stained for γ-H2AX (G) or p-p53 (I) and insulin. Representative images (G and I) and

Figure 6. *Continued*

quantitative percentage of γ-H2AX or p-p53-positive β-cells (H and J) are shown. Scale bars represent 50 μm. Data are pooled from three human islet preparations except for J (n = 2). (L) Representative Western blot of p-p53 protein is shown. All lanes are from the same gel but were run noncontiguously.

Data show means ±SEM. *$p < 0.05$ stimuli treated compared with control conditions. **$p < 0.05$ inhibitor-treated compared with treated conditions.

prevent cellular mutations and related abnormalities that contribute to tumorigenesis. However, DDR dysregulation leads to multiple pathological settings: extensive impairment of DDR leads to cancer with uncontrolled cell overgrowth, whereas extensive upregulation may precede neurodegenerative and metabolic diseases with specific cell loss (Jackson and Bartek, 2009; Shimizu et al., 2014). Several lines of evidence support the important function of USP1 in DDR processes (Cukras et al., 2016; Nijman et al., 2005; Ogrunc et al., 2016; Sourisseau et al., 2016). As DDR markers are highly elevated in human and rodent diabetic islets/β-cells *in vitro* and *in vivo* and correlate with β-cell death (Belgardt et al., 2015; Himpe et al., 2016; Nyblom et al., 2009; Oleson et al., 2014, 2016; Tornovsky-Babeay et al., 2014), we sought to determine whether USP1 inhibition would modulate DDR under diabetic conditions. Histone H2AX is a histone H2A variant, which is essential for cell cycle arrest and activation of DNA repair processes upon double-stranded DNA breaks. Upon DNA damage, H2AX is rapidly phosphorylated at Ser139 by PI3K-like kinases, including ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR), and DNA-dependent protein kinase (DNA-PK), and its phosphorylation generally referred to as γ-H2AX is universally considered as robust readout of DDR (Yuan et al., 2010). Consistent with previous studies (Belgardt et al., 2015; Himpe et al., 2016; Nyblom et al., 2009; Oleson et al., 2014, 2016; Tornovsky-Babeay et al., 2014), glucolipotoxicity and pro-inflammatory cytokines strongly induced DDR (represented by γ-H2AX formation; Figures 6A and 6B) in INS-1E β-cells. USP1 knockdown significantly blocked γ-H2AX upregulation, correlating with its anti-apoptotic action in response to diabetogenic stimulation (Figures 6A and 6B). Consistently, both USP1 inhibitors ML323 and SJB2-043 markedly reduced γ-H2AX in all tested diabetic conditions (Figures 6C–6F). To confirm β-cell-specific regulation of DDR by USP1 inhibition in human islets, we quantified the number of γ-H2AX-positive β-cells in human islets treated with the diabetogenic milieu in the absence and presence of ML323 and SJB2-043. Glucolipotoxicity induced a number of γ-H2AX positive β-cells, whereas USP1 inhibitors significantly reduced it (Figures 6G and 6H), suggesting the β-cell-specific suppression of γ-H2AX in human islets. Transcription factor p53 is activated in response to several cellular stresses, including DNA damage, and plays a key function in coordinating cell-intrinsic responses to exogenous and endogenous stressors by promoting cell cycle arrest, senescence, and apoptosis (Meek, 2009; Reinhardt and Schumacher, 2012). P53 phosphorylation at serine 15 is the primary event during DDR (phosphorylated by DDR-related kinases ATM and ATR), which promotes both the accumulation and functional activation of p53 and is the surrogate readout of DDR activation (Loughery et al., 2014; Meek, 2009; Meek and Anderson, 2009). To further test the efficacy of USP1 inhibition to suppress DDR, we quantified the number of p-p53 (at Ser15)-positive β-cells in isolated human islets treated with USP1 inhibitors exposed to the diabetic conditions. The number of p-p53-positive β-cells increased in glucose/palmitate-treated human islets, whereas both ML323 and SJB2-043 reduced such p-p53-positive cells (Figures 6I and 6J). Also, chronically elevated glucose-induced p-p53 upregulation was strongly inhibited by USP1 knockdown in INS-1E cells (Figure 6K). In addition, glucose/palmitate-induced p53 phosphorylation was fully blunted in the presence of SJB2-043 (Figure 6L) in human islets, further supporting the attenuation of DDR markers in diabetic β-cells by USP1 inhibition.

USP1 Inhibition Improves Survival and Lowers DDR in Diabetic β-cells

A progressive impairment of β-cell function together with increased β-cell death under diabetic conditions has been clearly documented in human β-cells. To test the potential beneficial effect of USP1 suppression in the human β-cell under conditions of T2D, we performed a proof-of-concept experiment, whereby we inhibited USP1 in two human islet preparations isolated from two patients with T2D. Islets were treated with ML323 and SJB2-043 for 24 hr. Consistent with the improved β-cell survival of human islets under diabetogenic conditions upon USP1 inhibition, isolated T2D islets treated with both USP1 inhibitors showed less apoptotic β-cells, demonstrated in two independent experiments from two different T2D human islet isolations (Figure 7A). In line with our data in INS-1E cells and human islets, both ML323 and SJB2-043 treatment highly reduced the number of γ-H2AX- and p-p53-positive β-cells in human islets consistently in two distinct human T2D islet batches (Figures 7B and 7C). This further confirms the USP1-dependent regulation of DDR under a diabetic environment.

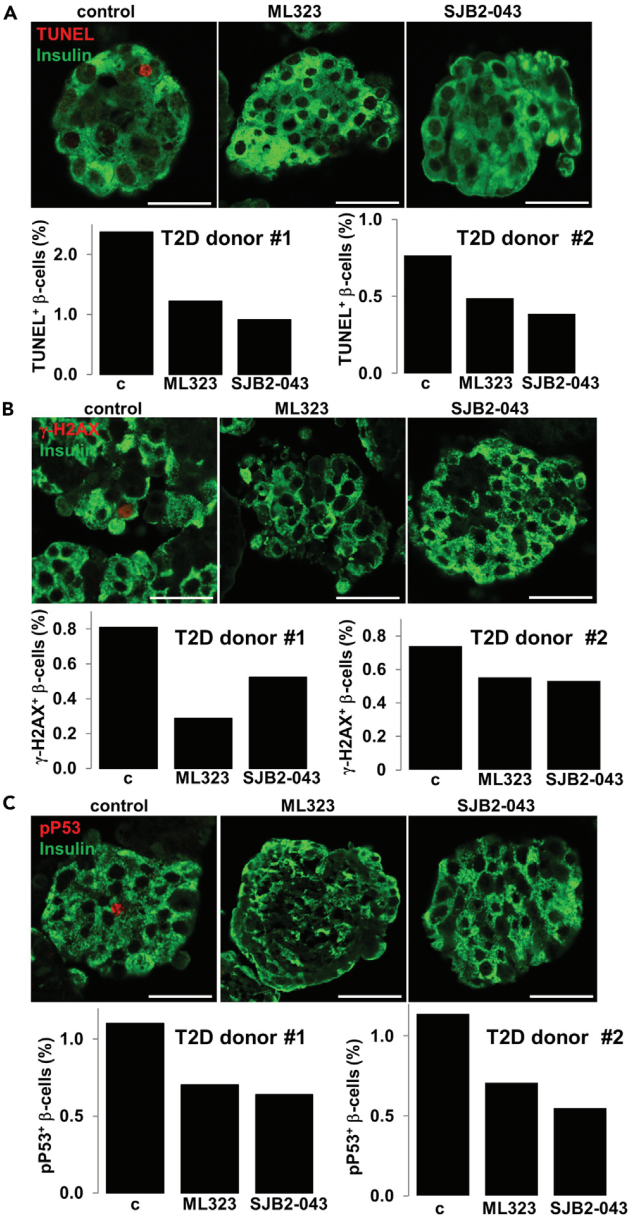

Figure 7. USP1 Inhibitors Reduced β-Cell Apoptosis and DDR in Human T2D Islets

Isolated human islets from two patients with T2D treated with or without USP1 inhibitors ML323 or SJB2-043 for 1 day. (A–C) Human pancreatic islets were fixed, paraffin embedded, and double stained for the TUNEL assay (A), γ-H2AX (B), or p-p53 (C) and insulin. Representative confocal images and individual quantitative percentage of TUNEL, γ-H2AX, or p-p53 positive β-cells are shown from 2 to 4 technical replica/group from two islet donors with confirmed T2D. Scale bars represent 50 μm. Data are means of TUNEL-, γ-h2AX-, or pp53- and insulin-co-positive β-cells from pooled data of 2–4 different human islet sections spanning the whole islet pellet for each experimental group. About 150–200 islets were plated for each group. The mean number of β-cells scored was 6,050 for each condition for each donor.

DISCUSSION

Loss of insulin-producing pancreatic β-cells results in hyperglycemia and is the hallmark of both T1D and T2D. Identification of key signaling molecules that promote β-cell death in diabetes, together with an understanding of their mechanisms of action, is critical for the disease pathogenesis as well as for novel therapeutic interventions to halt β-cell failure during development and progression of diabetes. This study

provides the first direct evidence that genetic or pharmacological inhibition of the enzyme USP1 protects β-cells from apoptosis under diabetogenic stimulation by attenuating DDR signals.

DDR is a signal transduction pathway, which functions together with other networking pathways known as DNA damage checkpoints (Harrison and Haber, 2006), and its dysregulation is a hallmark of several pathological disorders, such as cancer and neurodegenerative and metabolic diseases (Jackson and Bartek, 2009; Shimizu et al., 2014). Elevated DDR has been observed in pancreatic islets/β-cells under increased cellular stress and metabolic demand *in vivo* or under diabetic conditions *in vitro*. Surrogate markers of DDR, such as γ-H2AX, p53, and P53BP1, are highly upregulated in primary islets and β-cells in response to oxidative and inflammatory assaults as well as in pancreatic islets from streptozotocin (STZ)-treated diabetic mice, leptin-receptor-deficient *dbdb*, and human T2D islets (Belgardt et al., 2015; Himpe et al., 2016; Nyblom et al., 2009; Oleson et al., 2014, 2016; Tornovsky-Babeay et al., 2014), suggesting that oxidative- or metabolic-mediated double-strand breaks in the DNA and downstream activation of p53 may be a key pathogenic element of β-cell stress under metabolic and inflammatory conditions. This is further supported by the elevated incidence of diabetes in individuals who received irradiation to the pancreas (de Vathaire et al., 2012; Meacham et al., 2009). Furthermore, oxidized DNA and p53 signaling are both highly upregulated in human T2D islets (Sakuraba et al., 2002; Tornovsky-Babeay et al., 2014). Initially, the DDR coordinates a transcriptional program with DNA repair and cell cycle arrest (Ciccia and Elledge, 2010); however, under sustained cellular stresses when DNA damage can no longer be repaired, the DDR initiates apoptosis (Roos and Kaina, 2013). DUBs play an important role in DDR. Although multiple numbers of DUBs are involved in DNA repair and the downstream process, USP1 is the first enzyme characterized as the key player in DDR (Jacq et al., 2013). USP1 is the DUB responsible for deubiquitination of monoubiquitinated FANCD2 (Nijman et al., 2005), an integral component of the Fanconi anemia (FA) DNA repair pathway and PCNA (Kee and D'Andrea, 2012). USP1 and its associated partner USP1-associated factor 1 (UAF1) play an important function in promoting DNA homologous recombination (HR) repair in response to DDR via distinct mechanisms: through FANCD2 deubiquitination (Nijman et al., 2005) or by interacting with RAD51 associated protein 1 (RAD51AP1; Cukras et al., 2016). It is possible that the interactions with these known USP1 targets directly lead to the reduced γ-H2AX described in our study. Also, USP1-dependent regulation of other signaling pathways such as AKT (Zhang et al., 2016; Zhiqiang et al., 2012) or upstream elements of the DDR, such as CHK1, a key kinase involved in DDR and DNA repair, which is also stabilized by USP1 (Guervilly et al., 2011), may affect the DDR output together with γ-H2AX formation (Bozulic et al., 2008; Surucu et al., 2008; Xu et al., 2010). Owing to the complexity of DDR signaling and multi-layer actions of USP1, there may also be dual and time-/concentration-/cell-system-dependent effects on γ-H2AX formation or on DDR in general, as an induction of γ-H2AX upon USP1 inhibition has also been observed (Olazabal-Herrero et al., 2016). Our results suggest that the genetic and pharmacological suppression of USP1 in rodent β-cell and human islets protected the cells from DNA-damage-induced cell death with preserving β-cell insulin secretion and β-cell key maturation genes, proposing the critical function of USP1 in the regulation of β-cell apoptosis under different diabetogenic conditions. However, pathway(s) responsible for the regulation of USP1 as well as the molecular mechanism by which USP1 regulates DDR in β-cells, especially in the diabetic environment, remains unknown and warrants further mechanistic investigations.

Although several USP1 targets, such as FANCD2 and PCNA, have been established in the context of DDR, the identification of novel USP1 substrates in other cellular processes will provide further insights on the action of USP1 in regulating cell survival and stress response. Recently, TANK-binding kinase 1 (TBK1), a key regulator of the innate antiviral immunity and maintenance of immune homeostasis, has been discovered as a novel substrate of USP1 (Yu et al., 2017). Yu et al. showed that USP1 functions as an important cellular enhancer of Toll-like receptor 3/4 (TLR3/4)-, retinoic acid-inducible gene I (RIG-I)-, and cyclic GMP-AMP synthase (cGAS)-induced antiviral signaling by TBK1 deubiquitination and regulation of its stability (Yu et al., 2017). TBK1 has a pleotropic function in the metabolic process, inflammation, and diabetes. Identified as an inflammatory kinase that targets the insulin receptor (Munoz et al., 2009), TBK1 regulates insulin-stimulated glucose uptake in adipocytes (Uhm et al., 2017). TBK1 levels are upregulated in obesity and diabetes, and the inhibition of TBK1 together with another inflammatory IKK-related kinase IKKε by the dual kinase inhibitor amlexanox reduces weight, fatty liver, and inflammation, as well as promotes insulin sensitivity in obese diabetic mice (Reilly et al., 2013) and presents a significant reduction in HbA1c in a subset of patients with T2D (Oral et al., 2017). As diabetogenic conditions, such as elevated palmitic acid, induce β-cell death and dysfunction at least in part through TLR4 signals (Eguchi et al., 2012; Saksida et al., 2012) upstream of TBK1, it is likely that the newly identified USP1-TBK1 axis affects viability, stress

response, and inflammation in β-cells as well as in other metabolically active tissues in metabolic and inflammatory contexts. This remains to be investigated.

Conclusion

Impaired β-cell survival is a key pathogenic element of pancreatic β-cell's insufficiency in both T1D and T2D. Here we demonstrated the anti-apoptotic action of USP1 inhibition through the regulation of DDR in several *in vitro* and *ex vivo* experimental models of diabetes in stressed β-cells in both rodent and human cells. The identification of the previously uncharacterized function of USP1 in the regulation of β-cell apoptosis may have a potential therapeutic relevance for the preservation of functional β-cell mass in diabetes. To warrant this, identification of β-cell-specific USP1 substrates, detailed mechanistic analyses, as well as the *in vivo* preclinical assessment of utility, efficacy, and side effects of currently available USP1 inhibitors are required in the near future.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and two figures and can be found with this article online at https://doi.org/10.1016/j.isci.2018.02.003.

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AUTHOR CONTRIBUTIONS

Designed and preformed experiments, analyzed data, and wrote the paper, K.G.; assisted to perform experiments, B.L., K.A., T.Y.; designed and supervised project and wrote the paper, A.A., K.M. All authors critically reviewed the manuscript for important intellectual content and approved the final version to be published.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Loss of Deubiquitinase USP1 Blocks Pancreatic β-Cell Apoptosis by Inhibiting DNA Damage Response

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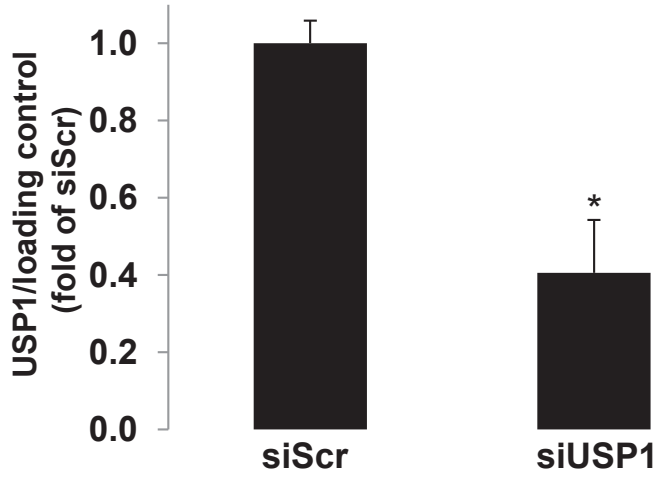

Figure S1. **USP1 knockdown, Related to Figure 1.** INS-1E cells were seeded at 300,000 cells/well and transfected with either control scrambled siRNA (siScr) or siRNA specific to USP1 (siUSP1). Quantitative densitometry analysis of USP1 protein level is shown. Data are pooled from at least three independent cell line experiments. Data show means ± SEM. *p<0.05 siUSP1 compared to siScr-transfected cells.

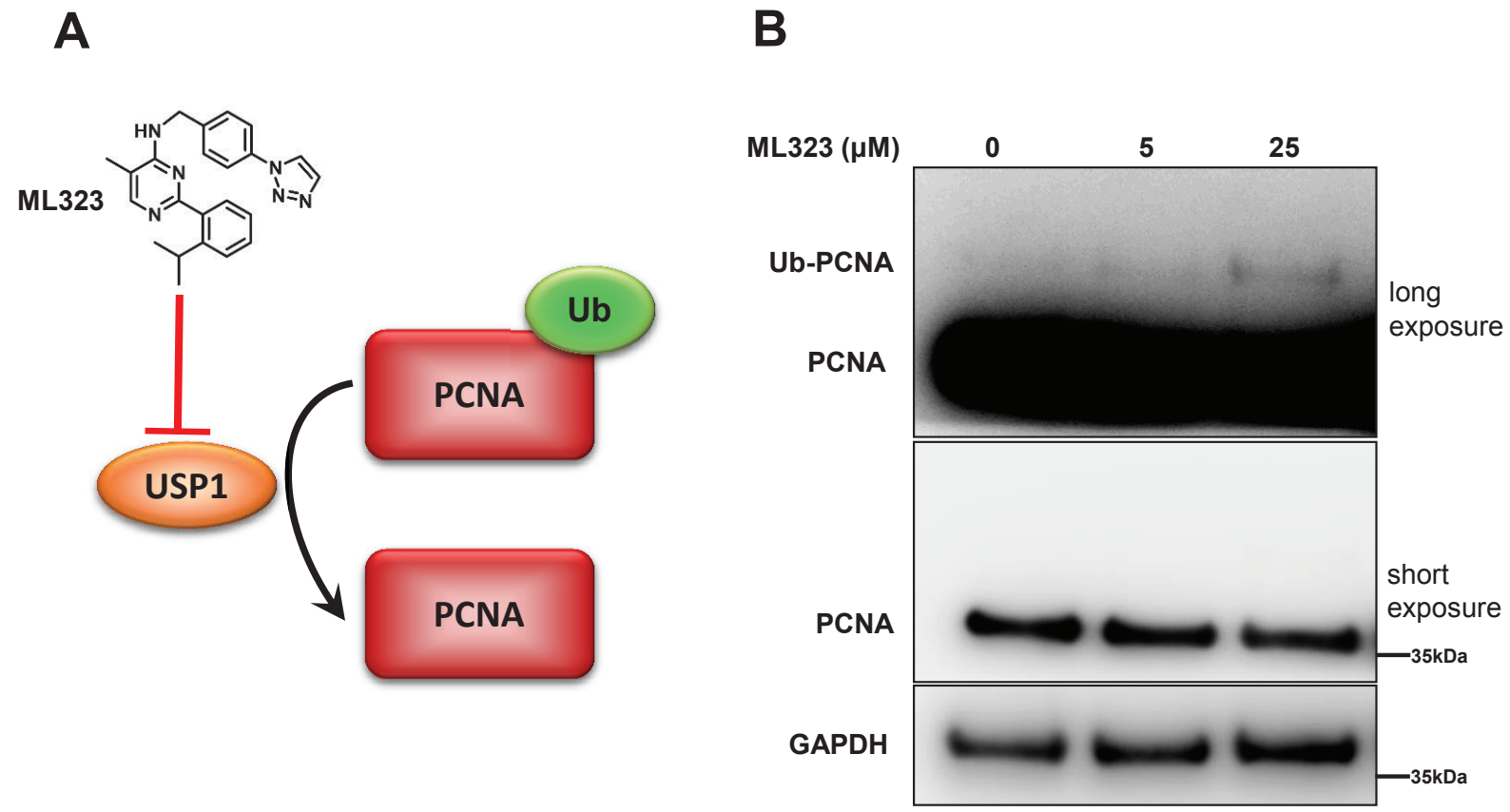

Figure S2. **Impact of the USP1 inhibitor ML323 on PCNA ubiquitination, Related to Figure 2.** (A) Schematic representation of USP1-mediated PCNA deubiquitination. (B) INS-1E cells were treated with USP1 inhibitor ML323 for 2 days. The same representative Western blot of PCNA is shown with long (upper panel) and short (lower panel) exposure time.

Transparent Methods

Cell culture, treatment, and islet isolation

Human islets were isolated from pancreases of healthy organ donors and from patient with type 2 diabetes at the University of Illinois at Chicago, Lille University and at ProdoLabs and cultured on extracellular matrix (ECM) coated dishes (Novamed, Jerusalem, Israel) as described previously (Schulthess et al., 2009). 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 and INS-1E cells in complete RPMI-1640 (Invitrogen) medium at 11.1 mM glucose as described previously (Ardestani et al., 2014). Islets and INS-1E were exposed to complex diabetogenic conditions, i.e. elevated glucose of 22.2 mM (Sigma), 0.5 mM palmitic acid (Sigma) and the mixture of cytokines: 2 ng/ml interleukin-1 beta (IL-1β), 1000 U/ml interferon gamma (IFN-γ) and 1000/ml tumor necrosis factor- alpha (TNF-α) (all R&D) (Ardestani et al., 2014). In some experiments, cells were additionally cultured with small molecule USP1 inhibitors ML323 (Axon Medchem), and SJB2-043 (ApexBio). Ethical approval for the use of islets had been granted by the Ethics Committee of the University of Bremen.

Transfections

To knockdown USP1 in INS-1E cells, the SMARTpool technology from Dharmacon was used. A mix of ON-TARGETplus siRNA against rat USP1 (100 nM, Dharmacon) was transiently transfected into INS-1E cells as described previously (Ardestani et al., 2014). An ON-TARGETplus non-targeting siRNA from Dharmacon served as control.

Western Blot analysis

Western blotting was performed as described previously (Ardestani et al., 2014). Membranes were incubated overnight at 4°C with rabbit anti-cleaved caspase-3 (#9664), rabbit anti-USP1 (#8033), rabbit anti-p-P53 (#9284), rabbit anti-γ-H2AX (#9718), rabbit anti-PARP (#9532), rabbit anti-cleaved PARP (rat specific #9545), mouse anti-PCNA (#2586), rabbit anti-tubulin (#2146), rabbit anti-GAPDH (#2118), and rabbit anti-β-actin (#4967) (all Cell Signaling Technology), followed by horseradish-peroxidase-linked anti-rabbit IgG (Jackson). Membranes were developed using a chemiluminescence assay system (Pierce) and analyzed using DocIT®LS image acquisition 6.6a (UVP BioImaging Systems, Upland, CA, USA).

Glucose-stimulated insulin secretion

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

RNA extraction and RT-PCR analysis.

Total RNA was isolated from cultured human islets using Trizol extraction system (TriFast-PEQLAB Biotechnology), and RT-PCR performed as described previously (Ardestani et al., 2014). For analysis, we used the Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, CA, USA) with TaqMan(R) Fast Universal PCR Master Mix for TaqMan assays (Applied Biosystems). TaqMan(R) Gene Expression Assays were used for *NEUROD1* (HS01922995_s1), *INS* (Hs02741908_m1), *PDX1* (Hs00236830_m1), *SLC2A2*

(Hs01096905_m1), *NKX2.2* (Hs00159616_m1), *NKX6.1* (Hs00232555_m1), and *PPIA* (Hs99999904_m1) as housekeeping control for all experiments.

Immunofluorescence

Isolated human islets were cultured for 24 or 48 hours under diabetogenic conditions with or without USP1 inhibitors and subsequently fixed in Bouin's solution for 15 min before embedding in paraffin as previously described (Sauter et al., 2008). Human 4µm sections were deparaffinized, rehydrated and incubated overnight at 4ºC with primary antibodies followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody or Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted with Vectashield with 4'6-diamidino-2-phenylindole (DAPI; Vector Labs). The following primary antibodies were used: rabbit anti-pP53 (Ser 15, #9284, 1:100, CST), or rabbit anti-γ-H2AX (Ser 139, #9718, 1:100, CST), and guinea pig anti-insulin (#A0546, 1:100, Dako). TUNEL staining for detection of apoptotic cells was carried out according to manufacturer's instruction (In Situ cell death detection kit, Roche). 4µm human sections were deparaffinized, rehydrated and incubated with TUNEL reagent for 1 hr afterwards with guinea pig anti-insulin (#A0546, 1:100, Dako) followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Slides were mounted with Vectashield with 4'6-diamidino-2-phenylindole (DAPI; Vector Labs). Fluorescence was analysed using a Nikon MEA53200 (Nikon GmbH, Dusseldorf, Germany) microscope and images were acquired using NIS-Elements software (Nikon). Confocal analyses were performed with an LSM 880 ZEISS confocal laser scanning microscope.

Statistical analysis

All values were expressed as means ± SEM with the number of independent individual experiments presented in the figure legends. The different groups were compared by Student's *t* test. P value<0.05 was considered statistically significant.

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3. Discussion

For a decade, protein degradation and stabilization have been identified as important mechanisms for regulating several cellular processes. The stability of most of the cellular regulatory proteins is controlled by the UPS; of which E3 ligases and deubiquitinases and their contribution to regulation of intracellular proteins homeostasis are most important [1]. The present study identifies a novel role for FBXO28, an important protein of the SCF ubiquitin ligase complex, in the regulation of β-cell survival in diabetes and the beneficial effects of the inhibition of deubiquitinase USP1 in protecting β-cells under diabetogenic conditions.

3.1. An SCF^{FBXO28} E-3 ligase protects β-cells from apoptosis

FBXO28 is an evolutionary, conserved nuclear F-box protein and a cell cycle directed protein which is involved in cell cycle progression and cellular processes [2, 3].

The important finding of my research is that FBXO28 protein levels are down-regulated in stressed β-cells and isolated human islets. The downregulation of FBXO28 is correlated with increased β-cell death in diabetic conditions (high glucose- or pro-inflammatory cytokines). It is well documented that chronic high glucose and free fatty acid levels increase reactive oxygen species (ROS) inside the pancreatic β-cells [4, 5]. Oxidative stress and ER stress resulted in the accumulation of misfolded and aggregative proteins in β-cells and lead to β-cell death. UPS plays a major role in the eradication of misfolded proteins to maintain β-cell homeostasis [6]. Prolonged oxidative stress and ER stress lead to dysregulation of UPS thus resulted in down-regulation of UPS components expression and function [7]. F-box proteins are known to play a crucial role in regulating ER stress through ERAD (ER-associated degradation) pathway. Misfolded proteins are eliminated by the ERAD pathway. F-box proteins recognize and ubiquitinate ERAD substrates, for 26S proteasome degradation [8, 9]. Therefore, F-box plays an important role in reducing the misfolded protein levels and thereby maintaining β-cell homeostasis. Such mechanisms could be an underlying cause of FBXO28 deficiency under diabetic conditions. However, such possibility needs to be investigated in the future.

In line with this, genetic depletion of FBXO28 induced β-cell death in basal conditions. Whereas, FBXO28 overexpression improved β-cell survival under diabetic conditions and reduced β-cell apoptosis. In line with earlier studies, depletion of FBXO28 resulted in dynamic loss of cell proliferation in normal human IMR90 cells and human diploid fibroblasts (HDFs) [3]. Additionally, inhibition of the proteasome resulted in high glucose-induced β-cell death in rodent β-cells and human islets [6]. In accordance with earlier research, my results show the important function of FBXO28 in cell survival.

β-Cell functional studies with FBXO28 revealed no effect on insulin secretion. Depletion of FBXO28 did not alter the insulin function suggesting that compensatory mechanisms for the FBXO28 loss are in place. FBXO28 overexpression regulated the expression of β-cell transcription factor NEUROD1 in isolated human islets, however, it did not alter the expression of several other tested β-cell identity and functional genes. NEUROD1 expression correlated with FBXO28 expression levels; FBOXO28 overexpression induced NEUROD1, while FBXO28 depletion significantly lowered NEUROD1 levels in human islets. My findings on β-cell survival with FBXO28 revealed, that FBXO28 is required for β-cell survival and is crucial in protecting β-cells from death under diabetes. The positive regulation of NeuroD1 with FBXO28 overexpression correlated with improved β-cell survival. Also, overexpression of FBXO28 did not restore β-cell function in islets from T2D patients. These data suggest, that despite its insignificant influence on β-cell function, FBXO28 plays a key role in β-cell survival. The UPS system regulation and function are very complex and F-box proteins are known to share the same substrate with different functions [2]. FbxW7 polyubiquitinated c-MYC for proteasomal degradation whereas non-proteolytic ubiquitination of c-MYC by FBXO28 resulted in activation of MYC-dependent transcription [2]. Further mechanistic experiments are required to reveal the mechanism.

Islet amyloid deposits lead to cell degeneration and amyloidosis has been related to a dynamic loss of pancreatic β-cell mass by apoptosis and is a pathological factor of T2D. [10-13]. Extracellular and intracellular amyloidosis was proven to cause ER stress and accumulation of ubiquitinated proteins [14, 15]. UPS might be vital for β-cell dysfunction in diabetes and for the clearance of such toxic islet amyloid deposits [11]. It is also reported that UPS is crucial for

insulin secretion by regulating specific proteins. The surface expression of molecules engaged in controlled insulin secretion like the ATP-sensitive potassium (K_{ATP}) channel [16] and the function of the voltage-dependent Ca^{2+} channel [17] relies on the ubiquitin-proteasome system. Subsequently, the proteasome may regulate a plenitude of functions of various proteins that direct the complex procedure of stimulated insulin secretion [17]. But, precisely how it is regulated or involved in insulin synthesis and secretion needs further evaluation [18].

According to a microarray analysis [19], FBXO28 is down-regulated in islets isolated from individuals with type 2 diabetes compared to nondiabetic individuals. But RT-PCR data in this work show that FBXO28 mRNA expression levels were not altered in human islets isolated from patients with T2D, compared to non-diabetic individuals. In contrast, FBXO28 protein levels were downregulated in β-cells under diabetogenic conditions, suggesting that FBXO28 as player of the cellular protein degradation system is itself rather regulated on the protein than on the mRNA level.

All together my findings suggest that FBXO28 is a pro-survival protein for the β-cell. Identifying β-cell specific substrates for FBXO28, its pathway establishment, and in vivo loss & gain of functional studies will provide further clues regarding functions and cellular actions of FBXO28 in pancreatic β-cells.

3.2. Loss of deubiquitinase USP1 blocks pancreatic β-cell apoptosis by inhibiting DNA damage response

While E3 ligases promote the conjugation of ubiquitin to target proteins, there are also several deubiquitinating enzymes (DUBs) that can reverse the ubiquitination process. Earlier studies underline the equal importance of E3s and DUBs and suggest that a tight balance between ubiquitination and deubiquitination is essential for cellular survival [20]. The control of ubiquitination and deubiquitination has a huge significance in cellular processes. DUBs dysregulations are linked to several pathological states. Thus, the DUBs have emerged as a novel targets in recent research (molecular targeting). DUBs regulate many pathways in cellular physiology. Dysregulation of DUBs triggers human disease conditions, such as several cancers,

immune pathways (NF-kB pathway and inflammasome) [21], heart diseases, Down's syndrome, also cellular process like cell cycle regulation, chromatin remodeling, differentiation, survival, DDR, and several signaling pathways (P53, receptor tyrosine kinase (RTK) Wnt, IFN, and Akt) [22-24]. The DUBs' potential to affect cellular processes, from signaling transduction and cell proliferation to apoptosis through altering ubiquitination and degradation of significant regulators is promising as well as exciting. DUBs are more likely drug candidates because of defined catalytic residues. Recently, DUBs have emerged as targets in developing novel therapies against several human malignancies. Presently available chemical inhibitors for inhibiting DUBs' activity are more encouraging and set the stage to develop selective and specific small molecule inhibitors for therapeutical usage [25]. Unrepaired DNA damage leads to cell death. Recently, upregulated DDR has been observed in pancreatic islets/β-cells under diabetic conditions [26]. DUBs are key players in altering DNA damage response. Although multiple numbers of DUBs are involved in DNA repair and its downstream process, ubiquitin-specific protease-1 (USP1) from the USP family is first characterized as a key player in DDR [27].

Knockdown of USP1 protects β-cells from apoptosis under diabetic conditions including prolonged culture with high glucose. Hyperglycemia contributes to the production of reactive oxygen species (ROS). The accumulation of ROS leads to oxidative stress of β-cells; in earlier studies, it's proven that the oxidative stress leads to DNA damage and results in DNA double-strand breaks (DSB). DSB leads to DNA damage, and single unrepaired DSB is sufficient to promote cell death [28]. My data strongly suggest that genetic depletion of USP1 or its inhibition with selective inhibitors (ML323 and SJB2-043) can rescue human β-cells and a rodent β-cell line from diabetogenic milieu induced DDR and β-cell apoptosis. However, USP1 inhibition does not alter the intracellular insulin content nor glucose-stimulated insulin secretion (GSIS). These observations confirm that USP1 inhibition has no direct effect on human islet insulin secretory function.

In pancreatic islets/β-cells upregulated DDR has been observed under increased cellular stress and metabolic demand in vivo or under diabetic conditions in vitro. The presence of proteins like 53BP1 and γH2AX in T2D β-cells evinces the DNA damage response. Recent research reports that glucotoxicity contributes to DNA double-strand breaks and activation of P53. It is also

reported that 53BP1 is recruited to sites of DSB whereas γH2AX foci on chromatin mark the DNA double-strand breaks [29-35]. Monoubiquitination of histone H2A at K119 is a crucial histone modification. Monoubiquitinated histone H2A and other histone variables (H2B, H3 & H4) play a central role in DNA damage response and repair pathways [20]. ATM kinase regulates the phosphorylation of H2AX at ser-139 by numerous phosphoinositide3-kinases (PIKKs) in response to DNA damage. Thus, γ-H2AX is a well-established universal marker of DDR signaling. P53 is another important marker for DDR activation. During DDR, P53 gets phosphorylated by DDR-related kinases ATM and ATR at serine 15. Phosphorylation of P53 at serine 15 is the prime event of DDR activation. Another important finding of my work is that USP1 knockdown or inhibition suppressed the DNA damage response (γ-H2AX and p-P53) in pancreatic β-cells and improved β-cell survival in isolated control and T2D human islets. Knockdown or inhibition of USP1 significantly blocked γ-H2AX upregulation in rodent β-cells, correlating with its anti-apoptotic action in response to diabetogenic stimulation. USP1 inhibition effectively counteracted the glucolipotoxicity induced DDR in isolated normal and T2D human islets as well as in rodent β-cells. This confirms a USP1-dependent regulation of DDR under a diabetic environment.

The USP1 specifically regulates the DNA-damage repair by preventing the monoubiquitination of FANCD2 and PCNA proteins. [27, 36]. The MRN (Mre11, Rad50, and Nbs1) complex senses the DNA DSBs (double-strand breaks), and recruits the transducers or DDR kinases belonging to the PIKK (Phosphatidylinositol-3-kinase-related kinase) family-ATM (Ataxia telangiectasia mutated) and ATM-Rad3-related (ATR) proteins, that are crucial in DNA damage response. This signal transduction targets transcription factors like P53, cell cycle regulators, the apoptotic machinery, and DNA repair factors including FANCD2 (Fanconi anemia D2) and PCNA (proliferating cell nuclear antigen) proteins [37]. The recruited transducer kinase ATM, with the help of MDC1 (Mediator for DNA damage checkpoint), BRCA1, 53BP1 (P53 binding protein1), ATR with TopBP1 (topoisomerase binding protein 1) and claspin mediator proteins activate effector kinases, such as Chk1 and Chk2. These activated effector kinases spread the signal all over the nucleus, which results in activation of multiple pathways including monoubiquitination of FANCD2 and PCNA proteins for DNA damage repair. Monoubiquitinated FANCD2 plays a crucial role in repairing the DNA damage by the interstrand cross-linking (ICL repair) pathway.

The monoubiquitinated FANCD2 relocates to nuclear DNA damage foci, to interact with BRCA1 and RAD51 and colocalizes with BRCA2 [38-40]**.** The monoubiquitinated PCNA recruits the TLS polymerases for lesion-bypass DNA synthesis and plays a crucial role in regulating the DNA damage by Translesion DNA synthesis (TLS).

Altogether, my findings strongly suggest that the USP1 inhibition has an anti-apoptotic action in stressed β-cells through the regulation of DDR. USP1 and its efficacy in regulating β-cell apoptosis could be a potential therapeutic target for the survival of functional β-cells in diabetes.

3.3. Conclusions

I have systematically studied two Ubiquitin-Proteasome System elements in pancreatic β-cells under physiological and diabetes-relevant conditions.

I have shown that:

- FBXO28 protein levels were lowered under diabetogenic conditions. While FBXO28 depletion induced β-cell death, its overexpression rescued β-cells from diabetes-induced apoptosis and improved β-cell survival.

- FBXO28 regulated the expression of β-cell transcription factor NeuroD1. FBXO28 modulation did not alter the insulin secretion in islets isolated from healthy controls and individuals with T2D.

- Genetic depletion of USP1 significantly reduced glucose, glucose/palmitate, and cytokine-induced apoptosis and promoted β-cell survival under diabetogenic conditions.

- Pharmacological inhibition of USP1 with highly potent selective inhibitors decreased apoptosis and thereby improved β-cell survival under diabetogenic conditions.

- USP1 inhibition in isolated human pancreatic islets fully preserved β-cell function and expression of β-cell functional and identity genes.

- USP1 inhibition significantly attenuated the DNA damage response in a rodent β-cell line, in normal and T2D human pancreatic β-cells. USP1 inhibition also lowered β-cell death in T2D.

3.4. Outlook

Despite a novel understanding of the Ubiquitin-proteasome pathway in pancreatic β-cell survival and function provided in the current study, many outstanding questions exist, which would be of great interest to further investigate in the near future:

1. The upstream regulators or an upstream signaling pathway that controls the level of FBXO28 protein especially under diabetic conditions.

2. The β-cell specific FBXO28 substrates need to be identified. Further studies on the physiological functions of the FBXO28 substrates in β-cell survival and function are required to understand the molecular mechanisms of FBXO28 action in β-cell biology.

3. β-cell specific FBXO28 transgene/knockout mice studies would provide further insights into the physiological regulation and function of FBXO28 at the cellular, molecular and organismic level in the control of metabolism.

4. Having established the anti-apoptotic effect of USP1 inhibition in β-cells and isolated human islets in vitro, it is necessary to investigate the impact of USP1 inhibition in pancreatic β-cell in terms of survival in vivo. β-cell specific USP1 knockout mice would provide further insights into the physiological role of USP1 in β-cells in vivo.

5. Identification of USP1 β-cell specific substrates, their physiological role in the context of β-cell survival, function, and DDR would reveal therapeutic insights in rescuing β-cell from DDR induced apoptosis towards diabetes treatment.

6. In depth in vivo experiments, full toxicology and pharmacology and pre-clinical studies with currently available USP1 inhibitors need to be performed in order to develop them as potential therapeutic molecules.

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4. Appendix

4.1. Reciprocal regulation of mTOR complexes in pancreatic islets from humans with type 2 diabetes

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

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ARTICLE

Reciprocal regulation of mTOR complexes in pancreatic islets from humans with type 2 diabetes

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Abstract
Aims/hypothesis Mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of nutritional status at the cellular and organismic level. While mTORC1 mediates beta cell growth and expansion, its hyperactivation has been observed in pancreatic islets from animal models of type 2 diabetes and leads to beta cell loss. We sought to determine whether such mTORC1 activation occurs in humans with type 2 diabetes or in metabolically stressed human islets and whether mTORC1 blockade can restore beta cell function of diabetic islets.
Methods Human islets isolated from non-diabetic controls and individuals with type 2 diabetes, as well as human islets and INS-1E cells exposed to increased glucose (22.2 mmol/l), were examined for mTORC1/2 activity by western blotting analysis of phosphorylation of mTORC1 downstream targets ribosomal protein S6 kinase 1 (S6K1), S6 and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and mTORC2 downstream targets Akt and N-myc downstream regulated 1 (NDRG1). mTORC1/2 complexes' integrity was assessed by immunoprecipitation and subsequent western blot analysis. Cell-type specific expression of activated mTORC1 in human islets was examined by immunostaining of pS6 (Ser 235/236) in human islet sections. Beta cell function was measured by glucose-stimulated insulin secretion (GSIS).
Results While mTORC2 signalling was diminished, mTORC1 activity was markedly increased in islets from patients with type 2 diabetes and in islets and beta cells exposed to increased glucose concentrations. Under high-glucose conditions in metabolically stressed human islets, we identified a reciprocal regulation of different mTOR complexes, with functional upregulation of mTORC1 and downregulation of mTORC2. pS6 immunostaining showed beta cell-specific upregulation of mTORC1 in islets isolated from patients with type 2 diabetes. Inhibition of mTORC1–S6K1 signalling improved GSIS and restored mTORC2 activity in islets from patients with type 2 diabetes as well as in islets isolated from diabetic *db*/*db* mice and mice fed a high-fat/high-sucrose diet.
Conclusions/interpretation Our data show the aberrant mTORC1 activity in islets from patients with type 2 diabetes, in human islets cultured under diabetes-associated increased glucose conditions and in diabetic mouse islets. This suggests that elevated mTORC1 activation is a striking pathogenic hallmark of islets in type 2 diabetes, contributing to impaired beta cell function and survival in the presence of metabolic stress.

Keywords Beta cells · Glucose · Human islets · mTORC1 · mTORC2 · Nutrients · Type 2 diabetes

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Abbreviations

4E-BP1	eIF4E-binding protein 1
eIF4E	Eukaryotic translation initiation factor 4E
GSIS	Glucose-stimulated insulin secretion
HFD	High-fat/high-sucrose diet
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex-1
mTORC2	mTOR complex-2
NDRG1	N-myc downstream regulated 1
S6K1	Ribosomal protein S6 kinase 1
siRNA	Small interfering RNA

Introduction

Pancreatic beta cells play a central role in controlling glucose homeostasis in response to metabolic fluctuations. The beta cells must coordinate multiple cellular metabolic processes to enable their insulin secretory responses to adapt to nutrient availability. Type 2 diabetes manifests itself by a progressive decline in beta cell functional adaptation and ultimate loss of beta cell mass by apoptosis [1]. The compensatory insulin response by pancreatic beta cells is a key rate-limiting step determining whether at-risk obese individuals develop diabetes. Nutrient overload, such as chronically increased glucose and NEFA concentrations, has been proposed as a main underlying pathological factor leading to beta cell deterioration during progression of type 2 diabetes [2]. Identifying the signalling mechanisms by which nutrient overload leads to impaired insulin action and beta cell death is instrumental for better understanding of the molecular changes underlying functional loss of beta cell mass and development of therapeutic strategies for the treatment of type 2 diabetes.

The mechanistic target of rapamycin (mTOR) is a master regulator of diverse cellular functions, such as metabolism, proliferation and survival, forming at least two functionally distinct complexes, mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2). mTORC1 phosphorylates various substrates, such as eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), S6 kinase 1 (S6K1) and SREBP, to promote anabolic processes and mTORC2 phosphorylates AGC kinases, including Akt and serum and glucocorticoid-regulated kinase 1 (SGK1), to enhance cell proliferation and survival. mTORC1 is a key nutrient sensor, integrating diverse extra- and intra-cellular cues to downstream signalling pathways in response to growth factors, stress, nutrient availability and other stimuli [3]. Considering the importance of metabolic control and mTORC1's role as a key sensor and regulator of cellular energy, it is not surprising that this signalling is dysregulated in a variety of disease states such as cancer and type 2 diabetes [3, 4]. mTORC1 activity is highly upregulated in the liver, fat, muscle and pancreatic islets of obese and high-fat-fed rodents [5–8]; this correlates with elevated circulating levels of insulin, pro-inflammatory cytokines and nutrients such as glucose and NEFAs and may represent a common consequential mechanism triggered by these driving forces in obesity. mTORC1 activity is constitutively elevated under conditions of nutrient satiation in the rodent beta cell line in vitro [9] and in the pancreatic islets of type 2 diabetic mice [8]. We therefore asked in the present study whether mTORC1 activity is altered in human isolated islets under conditions of metabolic stress and diabetes and whether acute inhibition of mTORC1 signalling can improve beta cell function.

Methods

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

Animals, islet isolation and culture Islets were isolated from high-fat/high-sucrose diet (HFD)-treated mice, leptin-receptor-deficient *Lepr*$^{db/db}$ (*db*/*db*) mice and non-diabetic heterozygous *Lepr*$^{db/+}$ (*db*/+) mice. Islets were cultured overnight in RPMI 1640 medium and treated with or without S6K1 selective inhibitor PF-4708671 for 4 h following protein isolation or glucose-stimulated insulin secretion (GSIS). See electronic supplementary material [ESM] Methods for details.

Transfections An adapted improved transfection protocol including Ca^{2+}-KRH medium was used to knock down raptor expression in human islets as described in detail in ESM Methods.

Western blot analysis Human islets and INS-1E cells were washed in ice-cold PBS and lysed in RIPA lysis buffer. Protein concentrations were determined with the BCA protein assay (Pierce, Thermo Fisher Scientific, Waltham, MA, USA).

Table 1 Human islet donor characteristics

Donor	Age (years)	BMI (kg/m^2)	Sex	HbA_{1c} (%)	HbA_{1c} (mmol/mol)	Cause of death
Type 2 diabetes	37	39.3	F	7.3	57	Anoxia
	48	41	M	6.5	48	Unknown
	56	24.3	M	5.1	32	Stroke
	54	29	M	6.5	48	Vascular/hypertension
	57	36.3	F	8.2	66	Cerebrovascular/stroke
	48	30.2	M	7.9	63	Trauma
	56	40	F	8.1	65	Cerebrovascular/stroke
	59	29.2	F	7.4	57	Anoxia, CPR
	51	24.4	M	6.9	52	Stroke
	58	39.3	M	8.9	74	Anoxia
	61	28	M	5.2	33	Cerebrovascular
Mean	53.2	32.9				
SEM	1.9	1.8				
Controls	23	24.5	F	4.9	30	Head trauma
	51	24.4	F	<6	<42	Head trauma
	31	23.8	M	<6	<42	Suicide (gunshot)
	48	25	M	<6	<42	Stroke
	54	37.3	F	4.9	30	Meningitis
	51	27.5	M	<6	<42	Unknown
	26	46.6	M	4.8	29	Head trauma
	56	33.1	F	<6	<42	Cerebrovascular/stroke
	82	22.2	F	<6	<42	Vascular event
	64	27.8	M	5.2	33	Stroke
	57	23.1	F	<6	<42	Stroke
	38	34	F	5	31	Anoxia
	51	24.1	M	<6	<42	Stroke
Mean	47.8	28.0				
SEM	4.3	1.8				

CPR, cardiopulmonary resuscitation; F, female; M, male

Equivalent amounts of protein from each treatment group were run on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electrically transferred onto polyvinylidene difluoride membranes. See ESM Methods for details.

Co-immunoprecipitation Immunoprecipitation was performed as described in detail in ESM Methods. Briefly, 500 μg of total lysate was incubated with rabbit anti-mTOR on a rotator at 4°C overnight. Immunocomplexes were then captured with Protein A Agarose Fast Flow (Millipore, Temecula, CA, USA) and were re-suspended in sample buffer and separated by NuPAGE 4–12% Bis-Tris gels (Invitrogen).

Immunofluorescence After isolation, human islets were processed for staining as previously described [11]. Sections (4 μm) were deparaffinised, rehydrated and incubated with primary antibodies overnight at 4°C, followed by FITC-conjugated secondary antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at RT. Slides were mounted with Vectashield with DAPI (Vector Labs, Burlingame, CA, USA). See ESM Methods for details.

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

Statistical analysis The experiments were not randomised and the investigators were not blinded to allocation during

experiments and outcome assessment. All values were expressed as means ± SEM with the number of independent individual experiments (biological replicates) presented in the figure legends. The different groups were compared by unpaired two-tail Student's *t* test; $p < 0.05$ was considered statistically significant.

Results

mTORC1 is hyperactivated in diabetic islets To examine the pathological relevance of mTORC1 activation in type 2 diabetes, we investigated whether mTORC1 activity was altered in islets isolated from patients with type 2 diabetes. Activation of mTORC1 was detected by phosphorylation analysis of its downstream targets S6K1 at Thr 389 (pS6K), the direct S6K substrate ribosomal protein S6 at Ser 235/236 (pS6) and 4E-BP1 at Thr 37/46 (p4E-BP1) (Fig. 1a). Western blots of pS6 and p4E-BP1 from human islets showed that mTORC1 activity was elevated in the islets from patients with type 2 diabetes (Fig. 1b). To thoroughly examine the activation of mTORC1 in control and diabetic islets, we analysed pS6 and p4E-BP1 signals by quantitative densitometry of immunoblots from ten non-diabetic and eight diabetic individuals (see Table 1). Our data showed that the mTORC1 activity was significantly increased in islets isolated from individuals with type 2 diabetes compared with the controls (1.76-fold increase in pS6; 3.07-fold increase in p4E-BP1; $p < 0.05$; Fig. 1c). To mimic a type 2 diabetes-like condition, islets isolated from humans were chronically cultured in elevated glucose concentrations for 72 h. Diabetes-associated elevated glucose profoundly upregulated mTORC1 activity in the islets (Fig. 1d, e), as demonstrated by increased phosphorylation of S6K1, S6 and 4E-BP1. Together, these data show that a diabetic milieu robustly upregulates mTORC1 activity in human islets.

Pancreatic beta cells are the source of upregulated mTORC1 in islets from individuals with type 2 diabetes As pancreatic islets are a composite of many cell types, including insulin-expressing beta cells and glucagon-expressing alpha cells, we aimed to determine whether increased mTORC1 activity is present in beta or non-beta cells. Cell-type-specific expression of activated mTORC1 in human islets was examined by immunostaining of pS6 (Ser 235/236). Immunofluorescent labelling of pS6 in combination

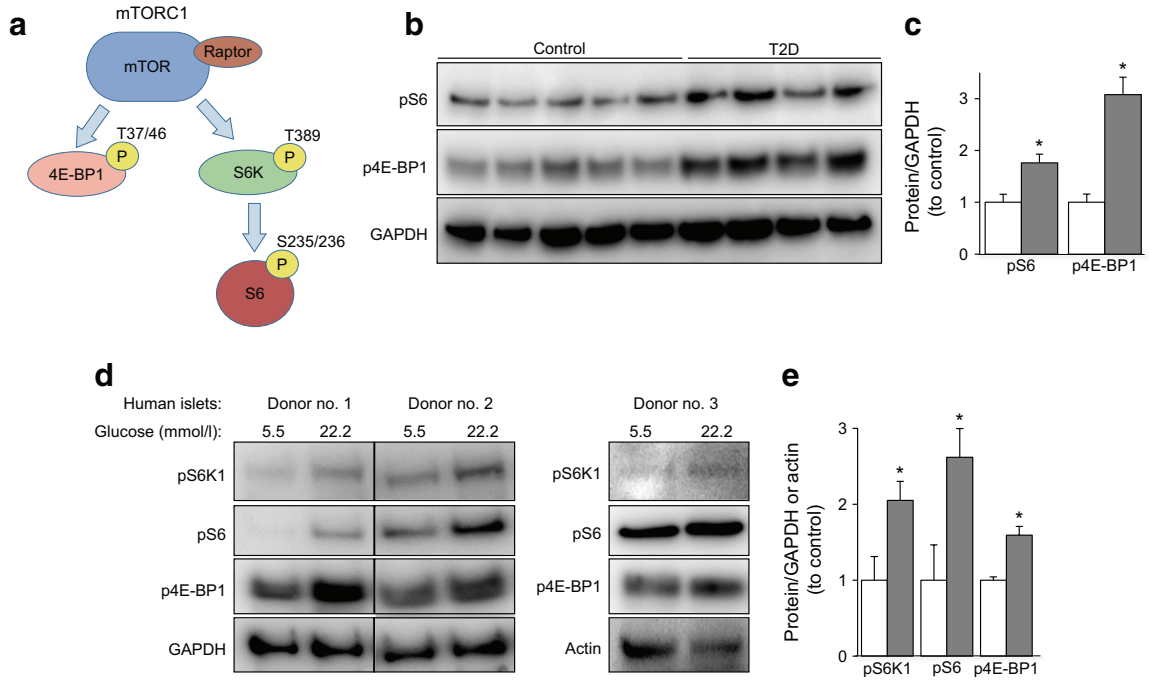

Fig. 1 mTORC1 is hyperactivated in diabetic islets. (**a**) Scheme depicting downstream targets of mTORC1. Activation of mTORC1 leads to phosphorylation and inactivation of 4E-BP1 (pT37/46) and activation of S6K (pT389). Active S6K phosphorylates its downstream target ribosomal protein S6 (pS235/236).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

with insulin or glucagon revealed higher mTORC1 activity in islets from individuals with type 2 diabetes. Activated S6 was present predominantly in insulin-expressing beta cells (Fig. 2a, c) and expression levels were very low in glucagon-expressing alpha cells (Fig. 2b, c). Quantification of insulin- or glucagon-co-expressing pS6 in sections of islets isolated from multiple human non-diabetic controls and individuals with type 2 diabetes (T2D) showed significant upregulation of activated S6 in beta cells and thus confirms higher mTORC1 activity in diabetic islets, indeed in the beta cell with insignificant expression in alpha cells (Fig. 2c). mTORC1 activity was also analysed in the established beta cell line INS-1E to provide comparison with primary human islets in terms of response to chronically elevated glucose concentrations. Prolonged culture of INS-1E cells with elevated glucose concentrations (22.2 mmol/l) led to robust upregulation of mTORC1, as represented by increased phosphorylation of S6K1, S6 and 4E-BP1 (Fig. 2d, e), confirming that mTORC1 hyperactivation indeed occurs in the beta cell.

Reciprocal regulation of different mTOR complexes in diabetic islets The activity and functional status of the two different mTOR complexes were examined by immunoprecipitation of endogenous mTOR and subsequent analysis of the mTOR-associated proteins raptor (representing mTORC1 activity) and rictor (representing mTORC2 activity). While endogenous raptor co-precipitated with mTOR (raptor-mTOR complex) was highly increased and confirmed higher activity of mTORC1 under high-glucose conditions, rictor co-precipitated with mTOR (rictor-mTOR complex) was drastically reduced upon prolonged culture of human islets with high glucose suggesting a reciprocal regulation of the two mTOR complexes in metabolically stressed human islets (Fig. 3a–c).

To confirm the functional decline of mTORC2 in diabetic islets, we investigated whether mTORC2 activity was changed in islets isolated from patients with type 2 diabetes. Activation of mTORC2 was assessed by phosphorylation analysis of its downstream targets Akt at Ser 473 (pAkt) and N-myc downstream regulated 1 (NDRG1) at Thr 346 (pNDRG1) (Fig. 4a). Consistent with the lower integrity of mTORC2 under high-glucose conditions, western blot analysis of pAkt and pNDRG1 from human islets showed that mTORC2 activity was diminished in the islets from patients with type 2 diabetes (Fig. 4b). To thoroughly examine the activation of mTORC2 in control and diabetic islets, we analysed pAkt and pNDRG1 signals by quantitative densitometry of immunoblots from eight non-diabetic and seven diabetic individuals. Our data showed that mTORC2 activity was significantly reduced in the diabetic islets compared with non-diabetic controls (77% and 53% reduction in pAkt and pNDRG1, respectively, $p < 0.05$; Fig. 4c).

mTORC1–S6K1 inhibition improves insulin secretion in islets from individuals with type 2 diabetes mTORC1 upregulation correlates with beta cell dysfunction in islets from individuals with type 2 diabetes [12, 13]. Knockdown of the critical component of mTORC1, raptor, is sufficient to increase insulin secretion in rodent beta cell lines [14]. Therefore, we hypothesised that mTORC1 inhibition would improve beta cell function in already-diabetic islets in the ex vivo setting. To date, no selective mTORC1 inhibitor has been discovered. Although rapamycin has widely been used to block mTORC1, it also inhibits mTORC2 [15–17]. Furthermore, the effects of rapamycin on islet biology are complex and controversial, with many laboratories reporting detrimental effects of rapamycin on beta cell function and survival [18]. Nevertheless, we tested the effect of different concentrations of rapamycin on islets from individuals with type 2 diabetes. Rapamycin at concentrations of 10 or 20 nmol/l had no effect on GSIS in diabetic islets (data not shown). This, as well as previous results, makes rapamycin an inappropriate approach by which to target mTORC1 in human islets.

As S6K1 is one of the major downstream effectors of mTORC1, we tested PF-4708671, a novel cell-permeable piperazinyl pyrimidine compound recently characterised as a selective S6K1 inhibitor [19], as an alternative approach for targeting mTORC1. The efficiency of PF-4708671 at blocking S6K1 signalling was confirmed in multiple human isolated islet preparations (Fig. 5c). Islets isolated from non-diabetic control individuals and from patients with type 2 diabetes were treated with 10 μmol/l PF-4708671 for 4 h. In line with previously published observations [20], the insulin content was drastically reduced in islets from individuals with type 2 diabetes when compared with non-diabetic controls (ESM Fig. 1). After treatment of non-diabetic and diabetic islets with S6K1 inhibitor for 4 h, there was no change in insulin content (ESM Fig. 1). While short-term treatment with inhibitor had no effect on GSIS in islets from non-diabetic individuals (Fig. 5a), it improved glucose-stimulated insulin response by 2.3-fold (with a strong tendency towards statistical significance [$p = 0.05$]), compared with the response in non-treated islets from individuals with type 2 diabetes. Such functional restoration occurred independently in all tested batches of human islets isolated from three patients with type 2 diabetes (Fig. 5b), indicating the beneficial effects of mTORC1–S6K1 signalling blockade.

While mTORC2 is critical for maintaining beta cell function [21], chronic hyperactivation of mTORC1 diminishes mTORC2–Akt signalling through multiple negative-feedback loops [22–24]. Thus, we examined whether mTORC1 inhibition can restore depleted mTORC2 in diabetic islets. In line with our insulin secretion data, S6K1 inhibitor increased mTORC2 activity as shown by increased phosphorylation of Akt (demonstrated independently in islets isolated from three donors with type 2 diabetes) (Fig. 5c, d). This

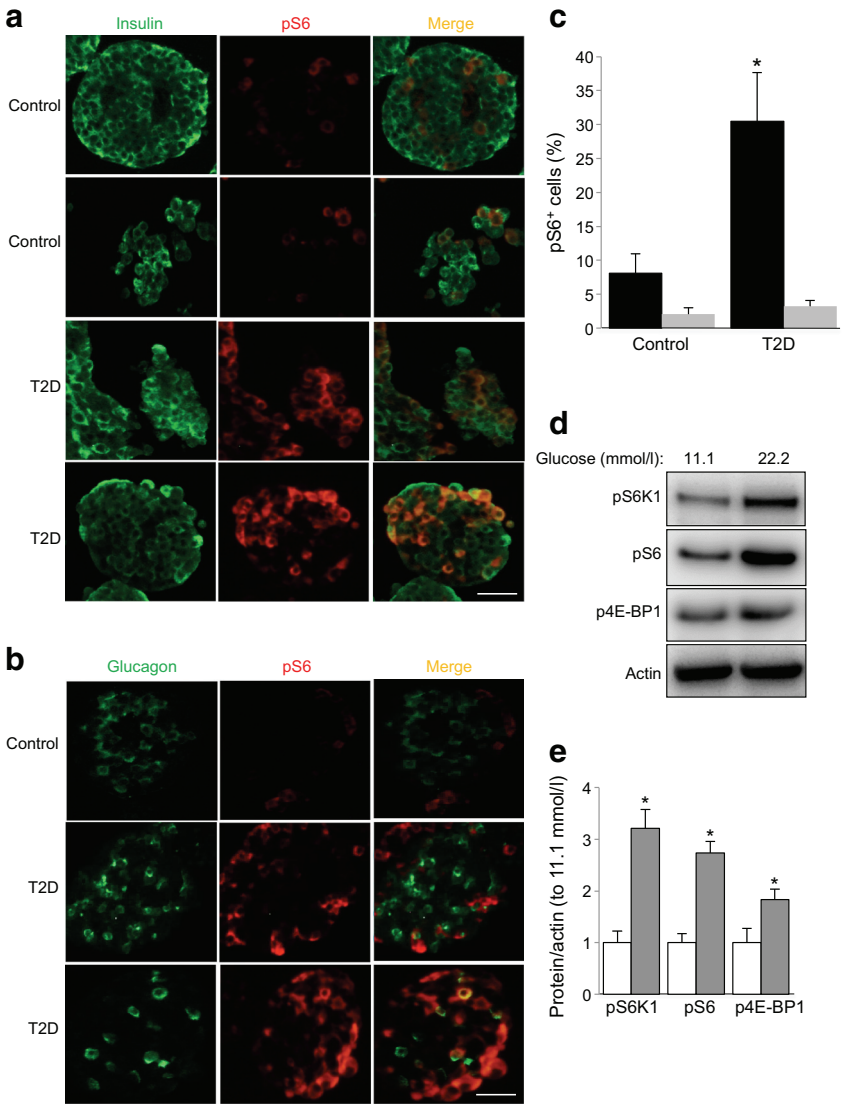

Fig. 2 mTORC1 is hyperactivated in diabetic beta cells. (**a**, **b**) Freshly isolated human islets from non-diabetic individuals as well as from patients with type 2 diabetes (T2D) were cultured overnight. Fixed paraffin-embedded islet sections were double-stained for pS6 (red) and insulin (green) (**a**) or glucagon (green) (**b**). Representative images from two non-diabetic control islets and two diabetic islets are shown. Scale bars, 100 μm. (**c**) Quantitative analysis of insulin- or glucagon-co-expressing pS6 in human islet sections from control and diabetic individuals. Data are means ± SEM from three non-diabetic controls ($n = 3$) and three individuals with T2D ($n = 3$). The number of cells that were counted for controls and T2D donors, respectively, was 2700 and 1725 insulin-positive beta cells and 1493 and 1585 glucagon-positive alpha cells. Black bars, pS6-insulin co-positive cells; grey bars, pS6-glucagon co-positive cells. *$p < 0.05$ compared with non-diabetic controls. (**d**, **e**) INS-1E cells were treated with 22.2 mmol/l glucose for 2 days. Representative western blots (**d**) and quantitative densitometry analysis of pS6K, pS6 and p4E-BP1 signals (**e**) are shown. Data are means ± SEM from three independent experiments ($n = 3$). White bars, control INS-1E cells; grey bars, high-glucose-treated INS-1E cells. Actin was used as loading control. *$p < 0.05$ compared with 11.1 mmol/l glucose-treated control INS-1E cells

suggests that successful inhibition of the mTORC1–S6K1 axis improved mTORC2 function most probably by restraining mTORC1-mediated negative-feedback loops.

To further corroborate the detrimental impact of hyperactivated mTORC1 on beta cell function in diabetic islets, we selectively inhibited mTORC1 by targeting its central component raptor. Targeted inhibition of endogenous mTORC1 by small interfering RNA (siRNA)-mediated silencing of raptor resulted in substantially increased GSIS (Fig. 5e, f) and efficiently reduced mTORC1 signalling (Fig. 5g), confirming the inhibitory action of upregulated human islet mTORC1 in type 2 diabetes.

S6K1 inhibition improves insulin secretion in isolated islets from obese diabetic mice A progressive decline of beta cell function leading to beta cell exhaustion, loss of compensatory beta cell adaptation, glucose intolerance and type 2 diabetes in response to long-term HFD have

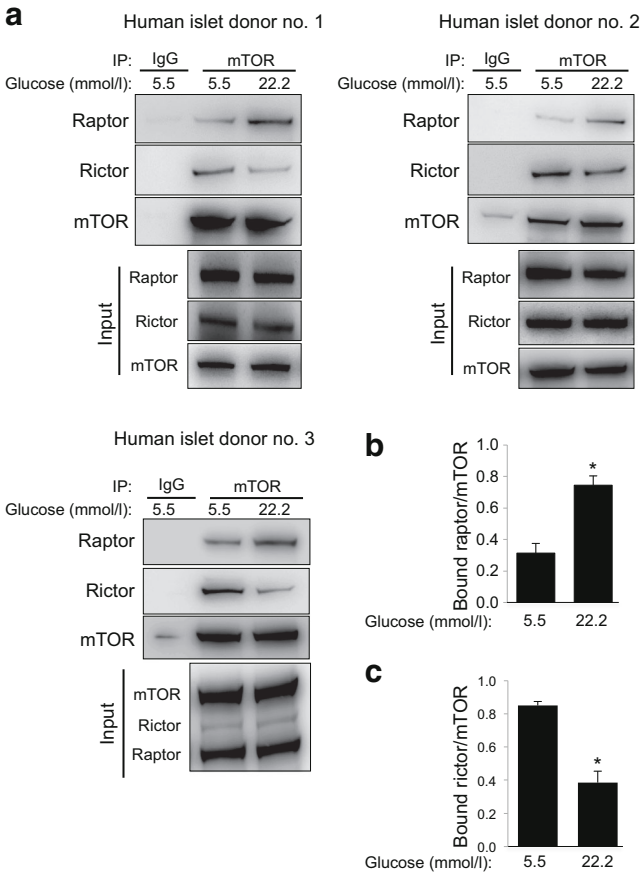

Fig. 3 Reciprocal regulation of mTORC1/2 complex integrity by chronically elevated glucose in human islets. (**a**) Human islets were treated with 22.2 mmol/l glucose for 3 days. Co-immunoprecipitations (IP) were performed using anti-IgG and anti-mTOR antibodies and western blots of precipitates and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

been clearly established in mice [10]. To further confirm the beneficial effect of mTORC1–S6K1 blockade on beta cell function, we examined whether acute S6K1 inhibition could improve insulin secretion in islets isolated from mice that had been treated with HFD for 17 weeks. Consistent with the improved beta cell function of human diabetic islets upon S6K1 inhibition, the diabetic mouse islets treated with S6K1 inhibitor showed significantly higher GSIS response compared with untreated HFD islets (Fig. 6a); the stimulatory index was highly improved (Fig. 6b). Intriguingly, and in line with human islet data, S6K1 inhibitor treatment greatly increased mTORC2 activity, as demonstrated by phosphorylation of Akt and NDRG1 (Fig. 6c, d). The efficiency of S6K1 inhibition in restoring mTORC2 activity was also tested in isolated islets from leptin-receptor-deficient *db*/*db* mice as another mouse model of type 2 diabetes. Inverse regulation of different mTOR complexes, with higher mTORC1 activity and lower mTORC2 activity, was reproduced in pancreatic islets of diabetic *db*/*db* mice (Fig. 6e). This confirms previously published observations in *db*/*db* mouse islets [8][25]. S6K1 inhibitor treatment fully blocked S6 phosphorylation and normalised mTORC2 activity in isolated *db*/*db* islets (Fig. 6e), confirming mTORC1–S6K1-mediated mTORC2 depletion as a possible pathogenic signalling hallmark of diabetic islets.

Discussion

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

Pancreatic beta cell-specific *Tsc2*-knockout mice, in which mTORC1 is constitutively active, show a biphasic pattern of beta cell turnover [8, 26]. While sustained mTORC1 activation leads to hyperinsulinaemia and improved glucose homeostasis through enhanced beta cell hypertrophy and hyperplasia in young mice, prolonged constitutive mTORC1 hyperactivation diminishes pancreatic beta cell mass by inducing apoptosis, leading to progressive hyperglycaemia and the development of diabetes in older mice. This cell-autonomous biphasic regulatory pattern of mTORC1 function can explain, at least in part, signalling alterations in the widely accepted model of beta cell deterioration 'compensation/decompensation switch' during the progression of type 2 diabetes. In the initial functional compensatory phase, metabolic drivers such as insulin resistance and nutrient excess increase beta cell mTORC1 activity; this correlates with higher insulin production as well as compensatory beta cell hypertrophy and hyperplasia, suggesting mTORC1 as a key positive

regulator of beta cell function and mass [27]. However, in the subsequent detrimental decompensatory phase, chronic activation of mTORC1 caused by sustained nutrient overload (mainly high glucose and NEFA) leads to beta cell exhaustion, functional collapse and ultimate cell death. The higher mTORC1 activity detected in the islets of individuals with type 2 diabetes in our study is consistent with observations made in animal models of type 2 diabetes and may be the consequence of nutrient overload in pancreatic beta cells.

How does chronic activation of mTORC1 promote impaired beta cell function and survival? It has been firmly established that mTORC1 mediates potent negative-feedback loops that restrain upstream mitogenic signalling from insulin/IGF receptor pathways [22–24]. These loops cause long-term compensatory mechanisms inhibiting Akt kinase via multiple mechanisms (e.g. mTORC1–S6K suppresses Akt signalling through phosphorylation of IRS1/2, Grb10 and Sin1 proteins [22–24]). Notably, chronic mTOR activation negatively regulates beta cell survival by direct IRS2 phosphorylation and subsequent proteasomal degradation [24]. Consistently, rapamycin-mediated mTORC1 inhibition upregulates IRS2 and Akt phosphorylation in mouse islets in vivo and in vitro, suggesting the presence of the negative functional loop from mTORC1 that prevents IRS2–Akt signalling [28]. A novel regulatory negative-feedback loop was established through mTORC1–S6K-mediated phosphorylation of the mTORC2 subunit Sin1 [23], inhibiting the mTORC2–Akt signalling axis, which is essential for maintaining normal beta cell mass [21]. This may explain the defective Akt-Ser473 phosphorylation (site of mTORC2 phosphorylation) seen in stressed beta cells [10, 25] and in islets from individuals with type 2 diabetes in our study and may be associated with the impaired mTORC2 complex integrity under prolonged glucose stimulation as presented here. Akt is the master pro-survival kinase in beta cells [10, 21, 27, 29]. One major detrimental outcome of the negative-feedback loops initiated by constitutive mTORC1 hyperactivity would be impaired Akt-mediated pro-proliferative and pro-survival responses. Indeed, defective Akt signalling is a hallmark of diabetic beta cells in the context of type 2 diabetes [10, 25]. Using various ex vivo experimental models of diabetes, including islets isolated from patients with type 2 diabetes as well as from hyperglycaemic HFD-treated and *db*/*db* mice, we demonstrated that inhibition of mTORC1–S6K1 signalling leads to striking restoration of mTORC2 activity; this suggests the existence of functional inhibitory loop(s) from mTORC1 to mTORC2. We speculate that such negative-feedback mechanisms triggered by mTORC1–S6K1 hyperactivation may operate in vivo in beta cells in type 2 diabetes. The identification of specific pathway(s) downstream of activated mTORC1, involved in the decline of mTORC2 signalling, may provide a better understanding of such complex interplay and some clues to its reversal.

Another so far unexplored but important mechanistic link between mTORC1 activity and beta cell failure in diabetes is the impairment of autophagy. Defective autophagy has been implicated in the process of beta cell failure in type 2 diabetes [26, 30, 31] and it is well established that mTORC1 hyperactivity results in inhibition of autophagy [26]. Mice deficient in autophagy-promoting protein 7 (Atg7), an essential component of autophagy, show exacerbated development of diabetes under high-fat diet, with

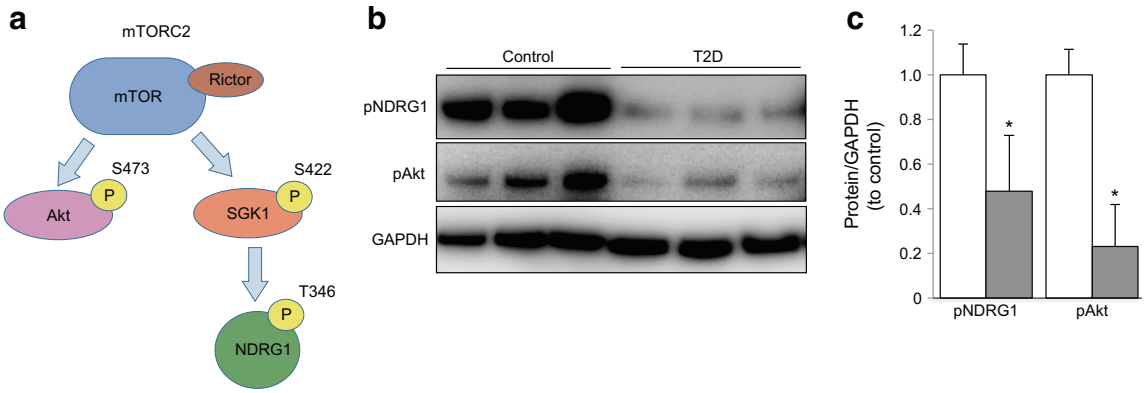

Fig. 4 mTORC2 activity is diminished in diabetic islets. (**a**) Scheme depicting downstream targets of mTORC2. Activation of mTORC2 leads to phosphorylation and activation of Akt (pS473) and SGK1 (pS422). Active SGK1 phosphorylates its downstream target NDRG1 (pT346). (**b**, **c**) Human islets isolated from eight non-diabetic controls ($n = 8$) and seven individuals with type 2 diabetes (T2D) ($n = 7$) were analysed for mTORC2 activity. pNDRG1 and pAkt were analysed by western blotting. Representative western blot (**b**) and quantitative densitometry analysis (**c**) of pNDRG1 and pAkt signals. GAPDH was used as loading control. White bars, control islets; grey bars, T2D islets. Data show means ± SEM. *$p < 0.05$ compared with non-diabetic controls

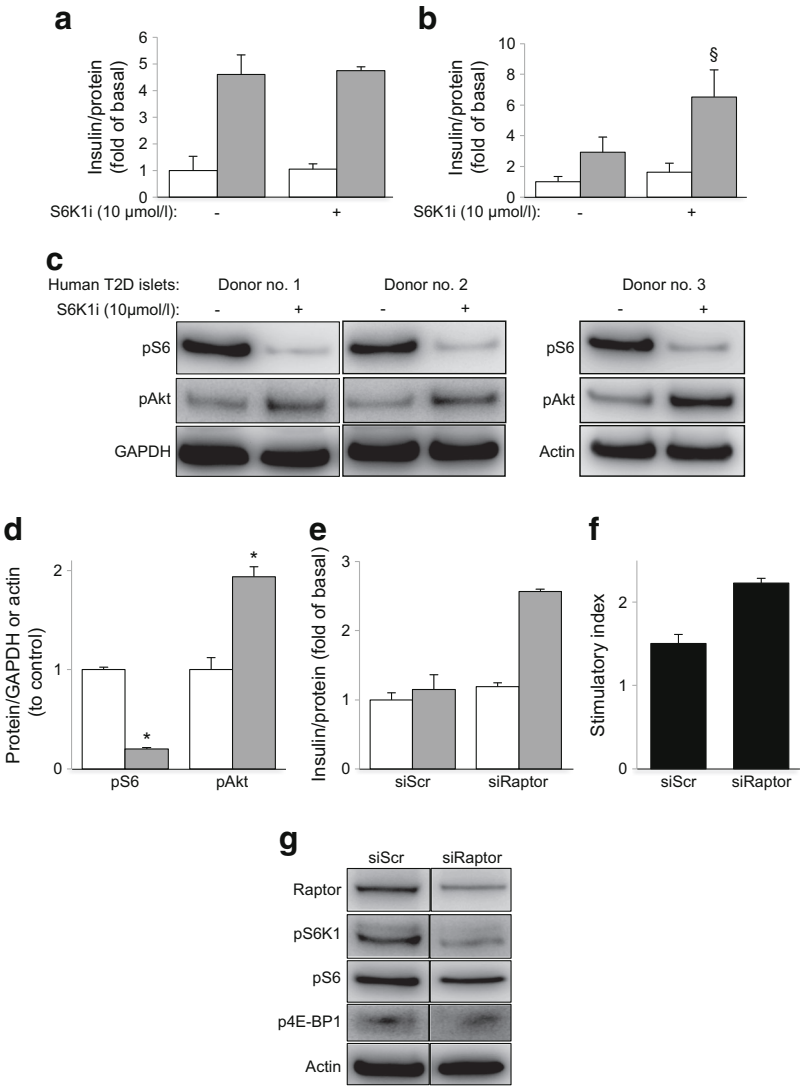

Fig. 5 S6K1 inhibition improves insulin secretion in islets from patients with type 2 diabetes. (**a**, **b**) Isolated islets from non-diabetic individuals (three donors; twelve to sixteen technical replicates per group) (**a**) and patients with type 2 diabetes (T2D) (three donors; eight to ten technical replicates per group) (**b**) were left untreated or treated with 10 μmol/l PF-4708671 (S6K1 inhibitor; S6K1i) for 4 h. Insulin secretion during 1 h incubation with 2.8 mmol/l (basal) and 16.7 mmol/l glucose (stimulated) was measured, normalised to protein content. White bars, basal insulin; grey bars, stimulated insulin. (**c**, **d**) Human islets isolated from three individuals with T2D ($n = 3$) were analysed for pS6 and pAkt. Western blots for three different human islets donors (**c**) and quantitative densitometry analysis of pS6 and pAkt signals (**d**) are shown. White bars, untreated human islets; grey bars, S6K1 inhibitor-treated human islets. (**e**–**g**) Islets isolated from patients with T2D (two donors; six technical replicates per group) were transfected with control (non-targeting) siRNA (siScr) or raptor siRNA (siRaptor). Insulin secretion during 1 h incubation with 2.8 mmol/l (basal) and 16.7 mmol/l glucose (stimulated) was measured (**e**), normalised to protein content. White bars, basal insulin; grey bars, stimulated insulin. The insulin stimulatory index (**f**) denotes the ratio of insulin secreted in response to 16.7 mmol/l to that secreted in response to 2.8 mmol/l glucose. Representative western blots of raptor, pS6K, pS6 and p4E-BP1 are shown (**g**). Both lanes were run on the same gel but were non-contiguous. Data show means ± SEM. $^{§}p = 0.05$ compared with S6K1 inhibitor-treated basal (**b**); $^{*}p < 0.05$ compared with untreated T2D islets (**d**)

diminished pancreatic beta cell mass and function [30]. Intriguingly, islets in type 2 diabetes accumulate autophagic vacuoles and autophagosomes concomitant with an increase in apoptotic beta cell death [32], suggesting an impaired flux of autophagy. In type 2 diabetes, the aberrant activation of mTORC1 in islets may provide a unique explanation for the chronic deterioration of the physiologically protective autophagy pathway during beta cell failure.

Despite the prevailing interest in mTORC1 inhibition for type 2 diabetes therapy, studies using mTOR inhibitors such as rapamycin show controversial results. While rapamycin

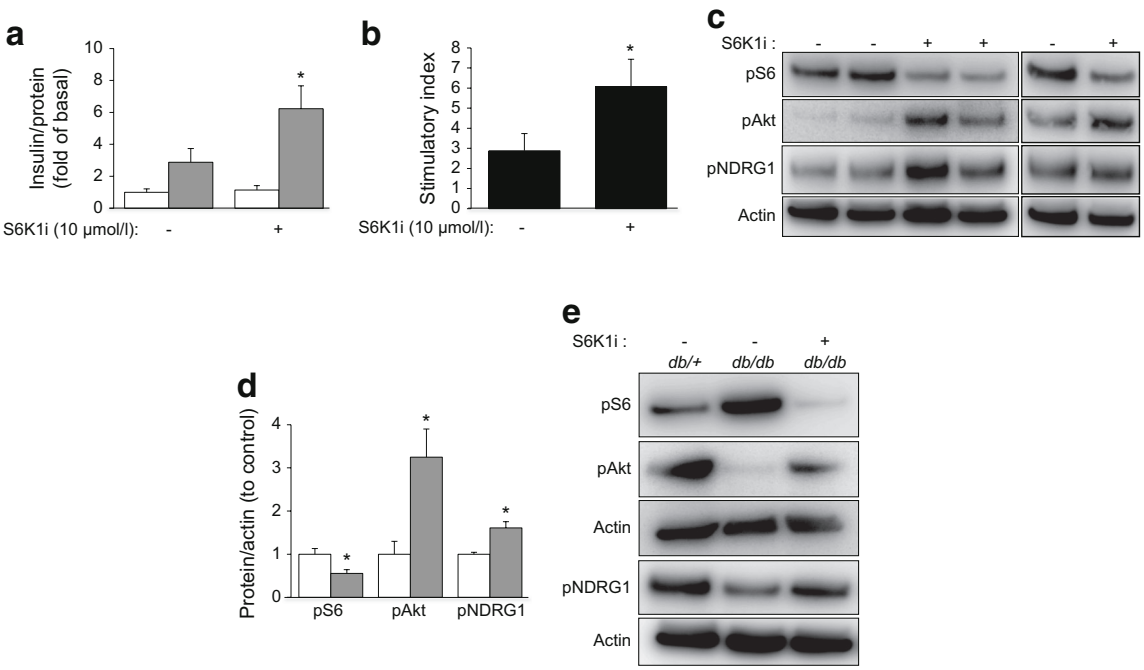

Fig. 6 S6K1 inhibitor improves insulin secretion in islets from diabetic mice. (**a**–**d**) Islets isolated from C57BL/6J mice that had been treated with HFD for 17 weeks were cultured overnight and then treated with or without 10 μmol/l PF-4708671 (S6K1 inhibitor; S6K1i) for 4 h. Insulin secretion during 1 h incubation with 2.8 mmol/l (basal) and 16.7 mmol/l (stimulated) glucose was measured (**a**), normalised to protein content. The insulin stimulatory index (**b**) denotes the ratio of insulin secreted in response to 16.7 mmol/l to that secreted in response to 2.8 mmol/l glucose ($n = 8$ per group). The isolated islets were analysed for pS6, pAkt and pNDRG1. Representative western blots of three different isolations (**c**) and subsequent quantitative densitometry analysis of pS6, pAkt and pNDRG1 signals (**d**) ($n = 3$) are shown. White bars, untreated HFD islets; grey bars, S6K1 inhibitor-treated HFD islets. (**e**) Islets isolated from diabetic 12-week-old *db*/*db* mice and their heterozygous non-diabetic littermate controls (*db*/+) were left untreated or were treated with S6K1 inhibitor for 4 h. Representative western blots of pS6, pAkt and pNDRG1 are shown. Data show means ± SEM. $^*p < 0.05$ compared with untreated HFD islets

suppresses stress-induced apoptosis in beta cell lines [9, 33] and improves beta cell function and thus corrects glucose homeostasis in the Akita mouse model of type 2 diabetes [34], chronic inhibition of mTORC1 by rapamycin causes glucose intolerance in mice [17, 28]. This is due to the fact that chronic inhibition of mTORC1 with rapamycin disrupts mTORC2, which is crucial for the insulin-mediated suppression of hepatic gluconeogenesis [17] and for maintaining pancreatic functional beta cell mass [21]. Alternatively, our data show that blocking mTORC1–S6K1 signalling using a highly specific S6K1 inhibitor enhances insulin secretion in islets from individuals with type 2 diabetes and from a mouse model of type 2 diabetes ex vivo, suggesting that the elevated mTORC1 activity seen in our study negatively regulates beta cell function. Interestingly, Shum et al [35] reported that rapamycin treatment compromised glucose metabolism and failed to improve Akt phosphorylation in liver, fat and muscle of HFD-treated mice and that, in contrast, S6K1 inhibition by PF-4708671 improved glucose tolerance and corrected HFD-induced impaired Akt phosphorylation in metabolically active tissues of obese mice. These previous data together with our study in human islets suggest the mTORC1–S6K1 axis as a potential therapeutic target for treatment of type 2 diabetes.

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Data availability All data generated or analysed during this study are included in this published article (and its supplementary information file).

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

Contribution statement AA conceived the project. TY, SR, KG, BL, AA and KM designed and performed experiments and analysed data. JO contributed reagents or analytical tools and contributed to interpretation of data. AA and KM wrote the paper and supervised the project. All authors critically reviewed the manuscript for important intellectual content and approved the final version to be published. AA and KM are the guarantors of this work.

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

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Dear Editor,

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

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

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

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

Conflict of Interest

The authors declare no conflict of interest.

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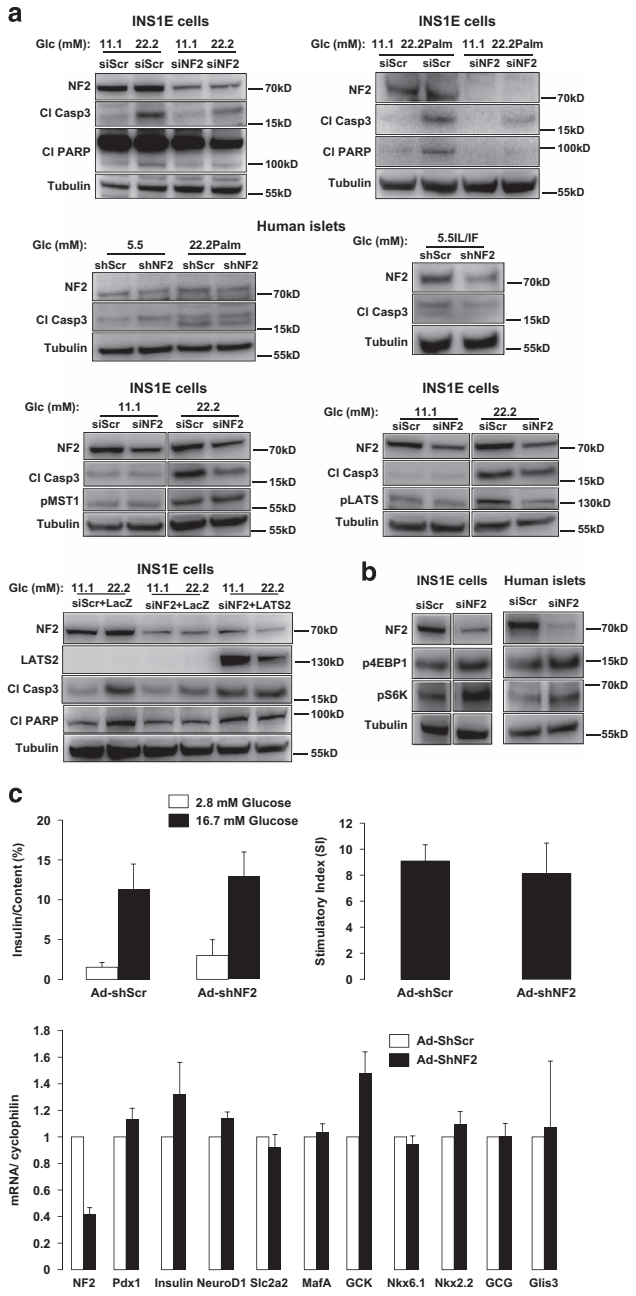

Figure 1 (**a**) INS-1E cells were transfected with either control Scr siRNA (siScr) or NF2 siRNA (siNF2) (both from Dharmacon, Lafayette, CO, USA) and treated with 22.2 mM glucose or the mixture of 22.2 mM glucose and 0.5 mM palmitate (22.2 Palm) for 2 days. Human islets were infected with either control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

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