The Hippo Terminal Effector YAP Potentiates Enteroviral Infection in Type 1 Diabetes

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1 Abstract

Type 1 diabetes (T1D) is a chronic autoimmune disease caused by the selective destruction of insulin-producing β-cells in the pancreas. Enterovirus infection (especially by the Coxsackie B virus family, CVB) implies a potential pathogenic mechanism triggering an autoimmune response against β-cells and exacerbated inflammation leading to β-cell destruction and T1D onset. While obesity associated Type 2 diabetes (T2D) also has a significant inflammatory component leading to β-cell destruction, a viral association has not been identified.

In order to verify whether there is a correlation between enterovirus infection and T2D, single molecule fluorescent in situ hybridization (smFISH) was used to detect enteroviral RNA in pancreases from organ donors with T2D. SmFISH is a molecular biology technique used to visualize and quantify the spatial distribution of RNA molecules within cells or tissues. It enables studying gene expression patterns at the single-cell level, providing insight into cellular heterogeneity and the organization of gene expression within tissues. We found Enteroviral RNA+ cells throughout the whole pancreas, but only few in the islets and numbers were similar in control pancreas and T2D donors. Although we observed increased islet lymphocyte infiltration and decreased β-cell area in T2D pancreases, there was no difference in the presence or localization of enteroviral RNA between control and T2D donors. No obvious pathological features of enterovirus infiltration were found in the pancreas in T2D, assuming that the onset of T2D to be less relevant to enteroviral infection than T1D.

The manipulation of the Hippo pathway by viruses highlights the complex interplay between viral infection and host signaling pathways. Viruses can modulate the activity of the Hippo pathway to exploit host cellular machinery either to promote their own replication and survival, but also to reduce an accelerated antiviral response. In the second part of my thesis, I investigated mechanisms of enteroviral infections in T1D. We found that Yes-associated protein (YAP), transcriptional coactivator and key downstream effector of the Hippo pathway, was highly upregulated in the exocrine and even in endocrine pancreas, where it is usually “disallowed”, of organ donors both with T1D-associated autoantibodies (AAb+) and with T1D. Most CVB-infected pancreatic cells either co-localized with YAP or were located near YAP-positive cells.

This observation assumes that the exocrine pancreas highly contributes to T1D progression. Overexpression of YAP promoted CVB replication, inflammation and further enhanced β-cell apoptosis. Mechanistically, inhibiting the interaction of YAP with its transcriptional regulator TEAD reduced the expression of YAP and YAP-induced replication of enteroviruses in pancreatic cells. Through in-depth analyses, I could clarify that YAP activated its own negative

regulator MST1 that constitutes a negative feedback loop to finally downregulate viral replication and β-cell death during CVB infections.

All in all, my work reveals the indispensable role of YAP as driver of enterovirus amplification in the pancreas. "Freezing" YAP in the pancreas constitutes a strategy to halt the process of enterovirus replication in the pancreas, slow down apoptosis and inflammation. A deep understanding of the regulatory role and mechanism of YAP in T1D is crucial for preventing β-cell failure and for the therapy of T1D.

1 Zusammenfassung

Typ 1 Diabetes (T1D) ist eine chronische Autoimmunerkrankung, die durch die selektive Zerstörung der insulinproduzierenden β-Zellen in der Bauchspeicheldrüse verursacht wird. Die Infektion mit Enteroviren (insbesondere der Coxsackie-B-Virus-Familie, CVB) stellt einen möglichen pathogenen Mechanismus dar, der eine Autoimmunreaktion gegen β-Zellen und eine verstärkte Entzündung auslöst, die zur Zerstörung der β-Zellen und zum Ausbruch von T1D führt. Während der mit Adipositas assoziierte Typ-2-Diabetes (T2D) ebenfalls eine signifikante Entzündungskomponente aufweist, die zur Zerstörung von β-Zellen führt, wurde ein viraler Zusammenhang nicht identifiziert.

Um zu überprüfen, ob es einen Zusammenhang zwischen Enterovirus-Infektion und T2D gibt, wurden Einzelmolekül-Fluoreszenz-in-situ-Hybridisierungen (smFISH) durchgeführt, um enterovirale RNA im Pankreas von Organspendern mit T2D nachzuweisen. SmFISH ist ein molekularbiologisches Verfahren zur Visualisierung und Quantifizierung der räumlichen Verteilung von RNA-Molekülen in Zellen oder Geweben. Sie ermöglicht die Untersuchung von Genexpressionsmustern auf Einzelzellebene und bietet so Einblicke in die zelluläre Heterogenität und die Organisation der Genexpression in Geweben. Wir fanden Enterovirus-RNA+-Zellen in der gesamten Bauchspeicheldrüse, aber nur wenige in den Inseln, und die Anzahl war bei Kontrollpankreas und T2D-Spendern ähnlich. Obwohl wir erhöhte Insel-Lymphozyten-Infiltration und verringerte β-Zell-Flächen im T2D Pankreas beobachteten, gab es keinen Unterschied in der Anwesenheit oder Lokalisierung von enteroviraler RNA zwischen Kontrollen und T2D Spendern. Es wurden keine offensichtlichen pathologischen Merkmale einer Enterovirus-Infiltration in der Bauchspeicheldrüse bei T2D gefunden, was vermuten lässt, dass enterovirale Infektionen in der Pathophysiologie und Entwicklung von T2D weniger relevant sind also für T1D.

Die Manipulation des Hippo-Signalwegs durch Viren zeigt das komplexe Zusammenspiel zwischen Wirtssignalwegen und viraler Infektion, und dass Viren die Aktivität des Hippo-Signalwegs modulieren und die zelluläre Maschinerie des Wirts nutzen können, um ihre eigene Replikation und Überleben zu fördern, aber auch entzündliche Reaktionen im Gleichgewicht zu halten. Im zweiten Teil meiner Arbeit untersuchte ich die Mechanismen von Enterovirus-Infektionen im T1D. Wir fanden heraus, dass Yes-associated protein (YAP), ein transkriptioneller Koaktivator und wichtiger Effektor des Hippo-Signalwegs, im exokrinen und endokrinen Pankreas, wo er eigentlich “unerlaubt” is und niemals auftritt, von Organspendern mit T1D-assoziierten Autoantikörpern (AAb+) und mit T1D stark hochreguliert war. Die

meisten CVB-infizierten Pankreaszellen waren entweder mit YAP kolokalisiert oder befanden sich in der Nähe YAP-positiver Zellen. Dies zeigt einen überraschenden Beitrag des Exokrinen Pankreas bei der Entstehung des T1D.

Die Überexpression von YAP verstärkte die CVB-Replikation und Entzündung sowie die Apoptose der β-Zellen. Mechanistisch gesehen reduzierte die Hemmung der Interaktion von YAP mit seinem Transkriptionsregulator TEAD die Expression von YAP und die durch YAP vermittelte Replikation von Enteroviren in Pankreaszellen. Weitreichend Untersuchungen zeigten, dass YAP seinen eigenen negativen Regulator MST1 aktivierte, der dann eine negative Rückkopplungsschleife bildet, um schließlich die virale Replikation und den Tod von β-Zellen während CVB-Infektionen zu verringern.

Alles in allem zeigt meine Arbeit die unverzichtbare Rolle von YAP als Treiber der Enterovirus-Vermehrung in der Bauchspeicheldrüse. Das "Einfrieren" von YAP in der Bauchspeicheldrüse stellt eine Strategie dar, um den Prozess der Enterovirus-Replikation zu stoppen und Apoptose und Entzündung zu verlangsamen. Ein tiefgreifendes Verständnis der Regulation von YAP im T1D ist entscheidend für die Verhinderung des Versagens der β-Zellen und für die Therapie des T1D.

Abbreviation

Aab	Autoantibody
AID	Auto inhibitory domain
AKT	Protein kinase B
ANKRD1	Ankyrin Repeat Domain 1
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
APCs	Antigen-presenting cells
cAMP	Cyclic adenosine monophosphate
CCL2	CC-chemokine ligand 2
CXCL8	CXC-chemokine ligand 8
COX-2	Cyclooxygenase-2
CREB	cAMP Response Element-binding
CTLs	Cytotoxic T lymphocytes
CRB-3	Crumbs-3
CVB	Coxsackievirus B
DCs	Dendritic cells
EGR-1	Early growth response-1
EPI	Exocrine pancreatic insufficiency
ER	Endoplasmic reticulum
FFAs	Free fatty acids
FOXA1	Forkhead box protein A1
GADA	Glutamic Acid Decarboxylase Antibodies
GAD65	Glutamic acid decarboxylase 65-kilodalton isoform
GDM	Gestational diabetes mellitus
GIP	Gastric Inhibitory Polypeptide
GLP-1	Glucagon-like Peptide-1
GLUT	Glucose transporter
GPCRs	G-protein-coupled receptors
HFMD	Hand, Foot, and Mouth Disease
HLA	Human leukocyte antigen
HSP	Heat shock protein
IAA	Insulin Autoantibodies
IA-2A	Insulinoma-Associated-2 Antibodies
IAPP	Islet amyloid polypeptide
ICA	Islet Cell Antibodies
IFN-1	Type I interferon
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL2RA	Interleukin 2 Receptor Subunit Alpha
IRF	Interferon Regulatory Factors
LATS1/2	Large tumor suppressor ½
LYP	Lymphoid tyrosine phosphatase

MALAT1	Metastasis associated lung adenocarcinoma transcript 1
MHC	Major Histocompatibility Complex
MOB1	MOB kinase activators 1
mRNA	Messenger RNA
MST1/2	Mammalian STE20-like protein kinase 1/2
NF-Kβ	Nuclear factor kappa β
NLR	Nucleotide-binding domain-leucine-rich repeat containing
NOD	Non-obese diabetic
PAMPs	Pathogen-associated molecular patterns
PDX-1	Pancreatic-duodenal homeobox gene 1
PDZ-BM	PDZ domain-binding motif
PKA	Protein kinase A
PKR	Protein kinase R
PRRs	Pattern recognition receptors
RASSF1A	Ras association domain family 1 Isoform A
RASSF6	Ras association domain family member 6
RLRs	RIG-like receptors
ROS	Reactive oxygen species
S100 A1	S100 calcium-binding protein A1
SARAH	Salvador-RASSF-Hippo
SAV1	Salvador homologue 1
SH3	Src homology domain 3
SH3-BM	Src Homology 3 binding motif
smFISH	Single molecule fluorescent in situ hybridization
STK38L	Serine/threonine kinase 38 like
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
T3cDM	Type 3c diabetes mellitus
TAD	Transcription activation domain
TAZ	Transcriptional coactivator with PDZ-binding motif
TBK1	TANK-binding kinase 1
TCR	T cell receptor
TEAD	TEA domain
TFAP2C	Transcription factor AP-2 Gamma
TG	Triglyceride
TID	TEAD transcription factor interacting domain
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
UCP2	Uncoupling protein 2
VP	Viral capsid protein
WBP5	WW domain binding protein 5
YAP	Yes-associated protein
ZnT8	Zinc transporter 8

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2 Introduction

2.1 Diabetes Mellitus, a global health and development challenge

Diabetes mellitus is an umbrella term used to describe a group of metabolic disorders characterized by high blood sugar levels over a prolonged period due to the patients' lack of the hormone insulin. Uncontrolled diabetes and sustained hyperglycemia is caused by β-cell failure and defective insulin secretion [1]. This condition of metabolic disease results in severe complications [2]. High blood glucose levels result in chronic damage and dysfunction of various tissues, especially the eyes, kidneys, heart, blood vessels, and nerves [3].

Medically, the main types of chronic diabetes are type 1 diabetes: caused by an absolute insulin deficiency due to autoimmunological destruction of the insulin-producing pancreatic β-cells [4] and type 2 diabetes: primarily initiated by obesity and systemic insulin resistance [5]. Currently, gestational diabetes mellitus (GDM) is classified as potentially reversible [6]. GDM is a complication during pregnancy, hyperglycaemia develops and may go away after the baby is born [7], but results in T2D later in life if the mother does not obey dietary restrictions and exercise. Other forms of diabetes are monogenic diabetes caused by mutations in a single gene, which affects insulin production or function [8]–[10], and secondary diabetes, a type of diabetes caused by certain medications, hormonal disorders, pancreatic diseases, or genetic syndromes [11].

Diabetes is a major and growing public health problem worldwide, affecting patients of all ages in both developed and developing countries [12], [13]. Based on WHO (World Health Organization) statistics, about 422 million people worldwide have diabetes. The International Diabetes Federation (IDF) estimates an increase to 693 million patients by 2045 if effective preventive measures are not taken [14]. Among the top ten causes of death in the world, diabetes greatly increases the mortality of patients with cardiovascular diseases, cancer, stroke and respiratory diseases [15], [16]. Overall, the global incidence of diabetes has increased significantly in recent decades and will continue to ascend [17]. Global public health and socioeconomic development will also face unprecedented pressure and challenges.

Disturbance in function and survival of insulin-producing pancreatic β-cells in the pancreas is the major cause of both T1D and T2D.

2.2 Pancreas and pancreatic β-cells

2.2.1 Pancreas structure and function

The pancreas is a large glandular organ that is located behind the stomach in the abdomen, surrounded by the small intestine, liver, and spleen. "Hidden" in the retroperitoneum, the pancreas, as the second largest digestive gland in the human body after the liver, plays a vital role in the digestive and endocrine systems [18]. Structurally, the pancreas has a unique elongated shape, consists with head, body, and tail [19], [20]. It measures about 16-18 centimeters long and weighs around 70 to 100 grams in adults [21]. The pancreas is composed of two main parts: the exocrine pancreas, which functions as digestive gland, and the endocrine pancreas, which is responsible for producing hormones that regulate blood sugar levels and glandular secretions [18], [22]–[24] (Figure 1).

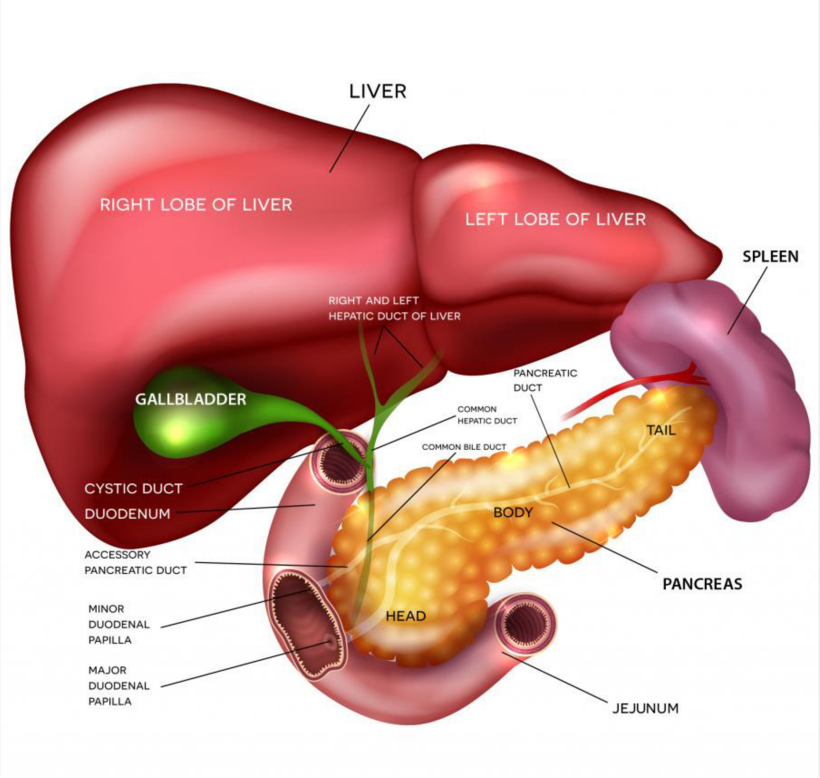

Figure 1. The location and structure of Pancreas. The head of the pancreas is located at the juncture where the first place that stomach meets the small intestine. The central part of the pancreas is called body, the first thin part followed head called neck which connect head and body. The last elongated part is the tail, and surrounded by spleen. Surround the pancreas several major blood vessels supplying blood to the pancreas and other abdominal organs, for example, the superior mesenteric artery, the superior mesenteric vein and the portal vein. (Adapted from https://kayebowers.com.au/pancreatic-cancer/).

Exocrine Pancreas:

The pancreatic exocrine gland is mainly composed of acinus and duct tissue, accounting for about 85-95% of the total weight of the pancreas [18]. It is mainly responsible for producing digestive enzymes including trypsin, chymotrypsin, carboxypeptidase, and elastase, which digest proteins and peptides into single amino acids; amylase and lactase, which digest carbohydrates into maltose, Maltotriose and dextrin; lipases, phospholipases, and esterases to break down fats; and nucleolytic enzymes that break down nucleic acids into mononucleotides and oligonucleotides [18], [25], [26]. Water and carbon dioxide are catalyzed by carbonic anhydrase to form carbonic acid (H_2CO_3), later through several times catalytic reaction into bicarbonate ions, the bicarbonate ions together with Na^+ ions and water later move through the intercalated ducts and at the end reach the main pancreatic duct ready for secretion into the duodenum [27]. In addition, pancreatic juice stimulates the gallbladder to produce bile, which, as an important digestive juice, is discharged into the duodenum together with pancreatic juice to help digest fats, carbohydrates, and proteins.

Endocrine Pancreas:

The endocrine pancreas consists of small bundles of cells called the islets of Langerhans, they are structurally arranged in a spherical shape. The endocrine tissue makes up only about 1-2% of the pancreas by weight, the number of cells exceeds a billion [26], [28]. The endocrine pancreas is the home from where the body regulates blood sugar levels, which are critical to maintain organ function and homeostasis. There are five major cell types which constitute the islet, α-cells secrete the hormone glucagon, which helps raise blood sugar levels by stimulating the liver to release stored glucose; β-cells secrete the hormone insulin, which works opposite to glucagon, mainly regulating blood sugar levels. Insulin helps lower blood sugar by facilitating the uptake and storage of glucose by cells; δ-cells secrete the hormone somatostatin, which helps regulate the release of insulin and glucagon; PP cells (F-cells) secrete the hormone pancreatic polypeptide, which is involved in various digestive processes and ε-cells, secrete the hormone ghrelin [29]–[31].

Exocrine pancreatic insufficiency (EPI) is one of the most common condition of the exocrine pancreas. It prevents the pancreas from producing enough pancreatic enzymes to help the body break down and absorb nutrients [18]. Hardt *et al.*'s study showed the increased susceptibility of patients with EPI to T1D as well as T2D [32]. Many studies confirm that the prevalence of EPI in patients with T1D is about 26-74% and the prevalence of EPI in patients with T2D is about 28-36% [32]–[36]. Most of the people thought that impaired insulin secretion is caused by dysfunction of the endocrine pancreas in typical diabetes (T1D & T2D). However,

another class of diabetes, namely pancreatogenic, pancreoprive or apancreatic diabetes, termed secondary or type 3c diabetes mellitus (T3cDM) has been overlooked [2], [37]. Strikingly, approximately 78.5% of T3cDM patients had chronic pancreatitis [38]. Therefore, it must be noted that in patients with diabetes, endocrine insufficiency can be caused by exocrine pancreatic dysfunction [39]–[41].

2.2.2 Pancreatic β-cells, Insulin Synthesis and Secretion

β-cells in the islets of Langerhans have a unique cellular structure that enables them to carry out their functions effectively. They possess a well-developed endoplasmic reticulum (ER), which is responsible for the synthesis and folding of insulin. This ER is known as the rough ER due to the presence of ribosomes attached to its surface [42], [43]. β-cells contain numerous insulin-containing secretory granules, which store insulin until it is ready for release [44]–[46].

Insulin synthesis involves a series of complex processes within pancreatic β-cells s, which begins with the transcription of the insulin gene (INS) into messenger RNA (mRNA) [47]. This process occurs in the nucleus of the β-cells (Figure 2A). Then the transcribed mRNA is translated into preproinsulin, a precursor molecule that contains a signal peptide [48], [49]. The signal peptide guides the newly formed preproinsulin to the ER. As preproinsulin enters the ER, the signal peptide is cleaved, resulting in the formation of proinsulin. Proinsulin consists of an A chain, a B chain, and a C peptide that connects them [49], [50]. Inside the ER, proinsulin undergoes folding, facilitated by chaperone proteins. During this process, disulfide bonds form between specific cysteine residues in the A and B chains of proinsulin. These disulfide bonds contribute to the three-dimensional structure of insulin. Proinsulin is transported from the ER to the Golgi apparatus. Within the Golgi, proinsulin undergoes further processing and packaging into secretory granules [48], [51]–[53]. Enzymes within the Golgi cleave the C-peptide from proinsulin, resulting in the formation of insulin and C-peptide. Insulin, along with C-peptide, is packaged into insulin granules [54], [55]. These granules are dense spherical structures that serve as storage compartments for insulin within the β-cells. The maturation and accumulation of insulin granules are regulated by factors such as glucose concentration, nutrient availability, and hormonal signals. These factors help control the number, size, and content of insulin granules within β-cells s. Upon appropriate stimulation, such as an increase in blood glucose levels, insulin granules undergo exocytosis. This process involves the fusion of the granule membrane with the cell membrane, releasing insulin into the extracellular space [55]–[58].

Disruption in insulin synthesis leads to abnormalities in insulin production, such as in diabetes mellitus. Understanding the mechanisms involved in insulin synthesis helps to shed light on the factors that influence its production and the regulation of blood sugar levels.

Insulin secretion from β-cells is tightly regulated and occurs in response to elevated blood glucose levels (Figure 2B). When glucose enters β-cells through glucose transporters (GLUT2), it undergoes metabolism, leading to the production of Adenosine triphosphate (ATP). ATP inhibits ATP-sensitive potassium channels, causing depolarization of the cell membrane [59], [60]. This depolarization opens voltage-gated calcium channels, leading to an influx of calcium ions [61], [62]. The rise in intracellular calcium triggers the exocytosis of insulin granules, releasing insulin into the bloodstream. In addition to glucose, other factors influence insulin secretion from β-cells (Figure 2C), such as Glucagon-like Peptide-1 (GLP-1) and Gastric Inhibitory Polypeptide (GIP). These incretin hormones are released from the intestine in response to food intake. They enhance insulin secretion from β-cells in the postprandial state [63], [64]. Somatostatin as an inhibitor of insulin secretion which is produced by delta cells within the pancreatic islets, helps regulate the release of insulin and glucagon, maintaining balanced blood glucose levels [65], [66]. In addition, the autonomic nervous system can modulate insulin secretion [67]. Parasympathetic stimulation generally enhances insulin release [68], [69], while sympathetic stimulation inhibits it [70], [71].

As the birth place of insulin production, β-cell mass, β-cell function and regenerative capacity are critical for insulin production and blood glucose regulation. However, in T1D, β-cells are selectively destroyed by an autoimmune response, resulting in absolute insulin deficiency. In T2D, insulin resistance prone to β-cells dysfunction.

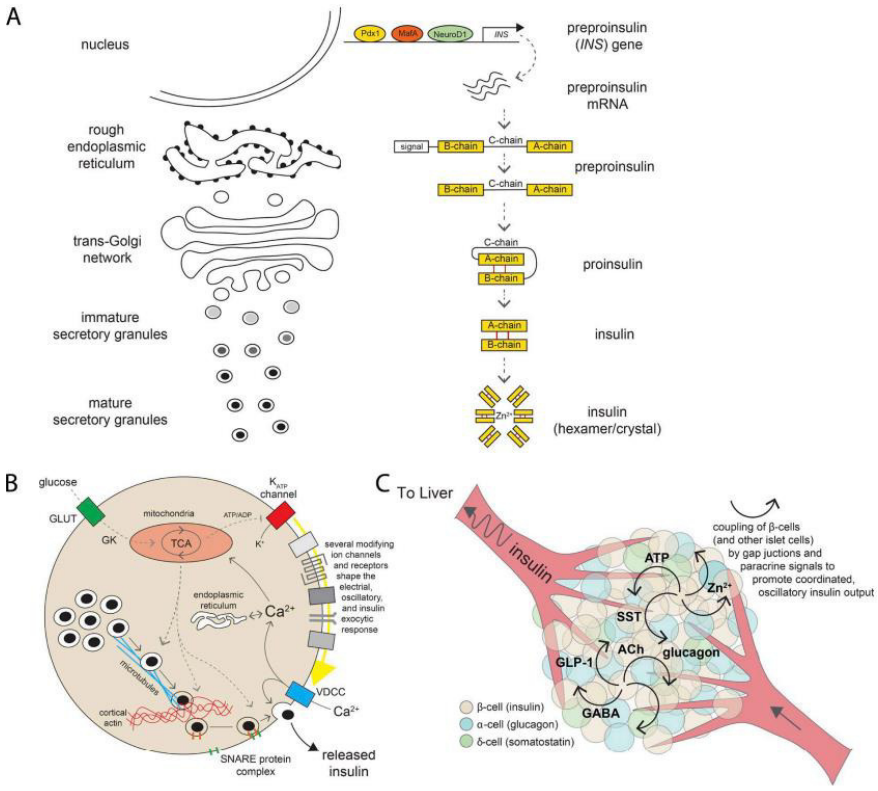

Figure 2. Insulin synthesis and secretion. The insulin gene is transcribe into mRNA in the β-cell nucleus. This mRNA contains the genetic instructions for building the insulin protein. The mRNA then leaves the nucleus and enters the cytoplasm complete translation. After translation, the initial insulin protein formed called preproinsulin. In the endoplasmic reticulum, preproinsulin formed to be proinsulin. Proinsulin then transported to the Golgi apparatus, where it is further modified and packaged into secretory vesicles. When stimulated, β-cells release insulin and C-peptide into the blood and play a role in regulating blood sugar levels. (Adapted from [54]).

2.3 Type 2 diabetes (T2D)

Type 2 diabetes mellitus is the most common form of diabetes, accounting for approximately 90% of diabetes cases. It is a chronic metabolic disease characterized by high blood sugar levels, also known as adult-onset diabetes or non-insulin-dependent diabetes [5], [72]. The pathogenesis of T2D involves a complex interplay between genetic and environmental factors [73]. Primary processes involved in the development of T2D include obesity, insulin resistance following impaired insulin secretion [72], [74], [75]. When the body is under insulin resistance situation, especially muscles, liver, and adipose tissue cannot respond as they should to insulin, glucose uptake is reduced [76]. When the β-cells fail to adapt to insulin resistance, hyperglycemia develops (Figure 3).

Similarly, insulin resistance can lead to reduced glycogen synthesis, further reducing glycogen storage capacity in the liver [77], [78]. Furthermore, insulin resistance attenuates the inhibitory effect of insulin on lipolysis, this result in an increase in the release of fatty acids from fat tissue into the blood, thus, elevated levels of circulating fatty acids further impair insulin signaling [79]–[81]. Leading to systemic lipid imbalances in other tissues and insulin resistance [82]–[84]. Dysregulation of adipokines secretion, in which adipose tissue produce and release more pro-inflammatory adipokines, these pro-inflammatory adipokines contribute to insulin resistance in adipose tissue and throughout the body [85]–[87].

Morever, Glucotoxicity and Lipotoxicity are playing crucial roles in the development of T2D. Long-term expose under high glucose, free fatty acids (FFAs), islet amyloid polypeptide (IAPP) and cytokines increase production of reactive oxygen species (ROS) induce ER stress and mitochondrial stress [43], affecting β-cell metabolism leading to β-cell dysfunction even death [88]–[90]. ER is primarily responsible for protein synthesis and folding, but is also involved in triglyceride (TG) and cholesterol synthesis and calcium homeostasis [91]. With the dysfunction of hypertrophic adipose tissue, increased lipolysis resulting in hyperlipidemia and excess FFAs. The latter combined with glucose overload resulted in increased activity of oxidative pathways. Over time, this energy overload can lead to mitochondrial dysfunction, which leads to increased ROS [92]. Mitochondrial dysfunction is associated with impaired peripheral tissue insulin responsiveness and β-cell secretory function and survival [93]. For example, the latter is regulated through pancreatic-duodenal homeobox gene 1 (PDX-1) a key regulator of insulin secretion β-cell survival [94]–[96] and mitochondrial uncoupling protein 2 (UCP2) [97]–[100]. Glucotoxicity and lipotoxicity have been implicated as mediators of ER stress, leading to disruption of ER Ca^{2+} homeostasis and inhibition of protein folding [101]. Mechanisms of glucotoxicity and lipotoxicity are intertwined and mutually reinforcing, leading to insulin resistance and impaired β-cell function, forming a vicious cycle.

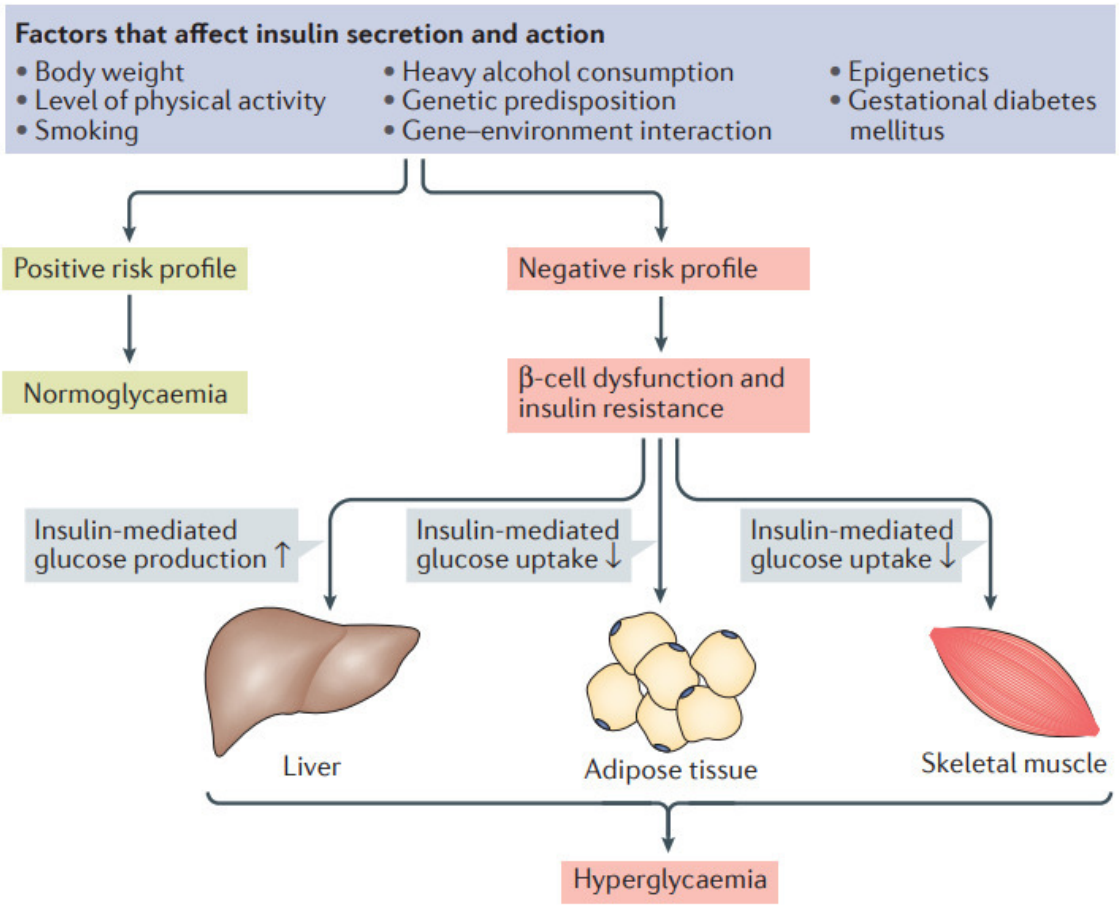

Figure 3. Pathophysiology of hyperglycemia in T2D. Obesity and a sedentary unhealthy lifestyle are listed at the top towards impaired insulin action and insulin secretion. Uncompensated β-cell dysfunction leads to an excessive amount of glucose circulating in the blood, causing hyperglycemia. (Adapted from [76]).

Furthermore, chronic low-grade inflammation is an essential feature during the development of T2D [102]. A possible link between inflammation and diabetes was first discovered more than a century ago when patients took high doses of sodium salicylate which impressively lowered blood sugar levels [103], [104]. Hotamisiligil *et al.* discovered that tumor necrosis factor-alpha (TNF-α) as a pro-inflammatory cytokine produced by adipocytes, can induce insulin resistance in animal models, and first revealed molecular pathways that link inflammation and insulin resistance [105].

In fact, the systemic inflammation associated with T2D involves multiple tissues and organs, especially the inflammation mediated by obesity contribute the risk of developing T2D.

Infiltration of macrophages and immune cells in adipose tissue from patients with obesity leads to local and systemic chronic low-grade inflammation. Hypoxia, adipocyte death, and increased secretion of chemokines and adipokines are thought to trigger immune cells and initiate their adipose tissue infiltration [106]. Obesity induces the expression of NF-κB target genes, such as pro-inflammatory cytokines, tumor necrosis factor (TNF), IL-6, and interleukin-1β (IL-1β) in the liver and adipose tissue [74], [107], [108]. Interestingly, TNF and IL-1β, which produced in response to NF-κB activation can also activate both JNK and NF-κB through the engagement of their specific cellular receptors [109]. TNF-α as an important factor involves in insulin resistance, insulin signaling regulation and insulin secretion [110]–[113]. Increased production and secretion of IL-1β is induced by glucotoxicity, blocking IL-1β signals protects the β-cell from IL-1β- and glucose-induced cell death [114], [115].

In addition, eutrophication can cause stress on pancreatic islets and insulin-sensitive tissues such as adipose tissue, leading to the local production and release of cytokines and chemokines, such as IL-1β, TNF, CC-chemokine ligand 2 (CCL2), CCL3 and CXC-chemokine ligand 8 (CXCL8) [109].

2.4 Type 1 diabetes (T1D)

T1D is a chronic autoimmune disease, which results from the activation of immune cells destroying the insulin-producing pancreatic β-cells leading to absolute deficiency of the hormone insulin, therefore it is also called insulin-dependent diabetes or autoimmune diabetes [116], [117]. Even though the proportion of people with T1D is less compared with the nearly 90 percent T2D, T1D occurs earlier, develops faster and with more severe complications. Distressingly, life expectancy is reduced by more than 20 years and usually begins in childhood and adolescence.

Although the etiology of T1D is not fully clear, its pathogenesis is thought to be initiated by specific T-cell-mediated destruction of pancreatic β-cells [118]. The immune system, particularly the adaptive immune system, plays a central role in this process (Figure 4). T-cells are key players in the autoimmune response [119], [120]. In individuals with T1D, T-cells ($CD4^+$ and $CD8^+$) mistakenly identify β-cells as foreign and launch an attack against them. β-cells are also destroyed by macrophages infiltrating the islets [119]–[121] and by inflammatory cytokines [4], [122]. This immune response leads to progressive β-cells destruction and the subsequent loss of insulin production [118].

Autoantibodies, including Islet Cell Antibodies (ICA), Insulin Autoantibodies (IAA), Glutamic Acid Decarboxylase Antibodies (GADA), Insulinoma-Associated-2 Antibodies (IA-2A), Zinc

transporter 8 (ZnT8), serve as markers of islet autoimmunity [123]–[126]. In T1D, these autoantibodies specifically target the insulin-producing β-cells in the pancreas [127]. They indicate the presence of an ongoing autoimmune process and serve as predictors of disease development.

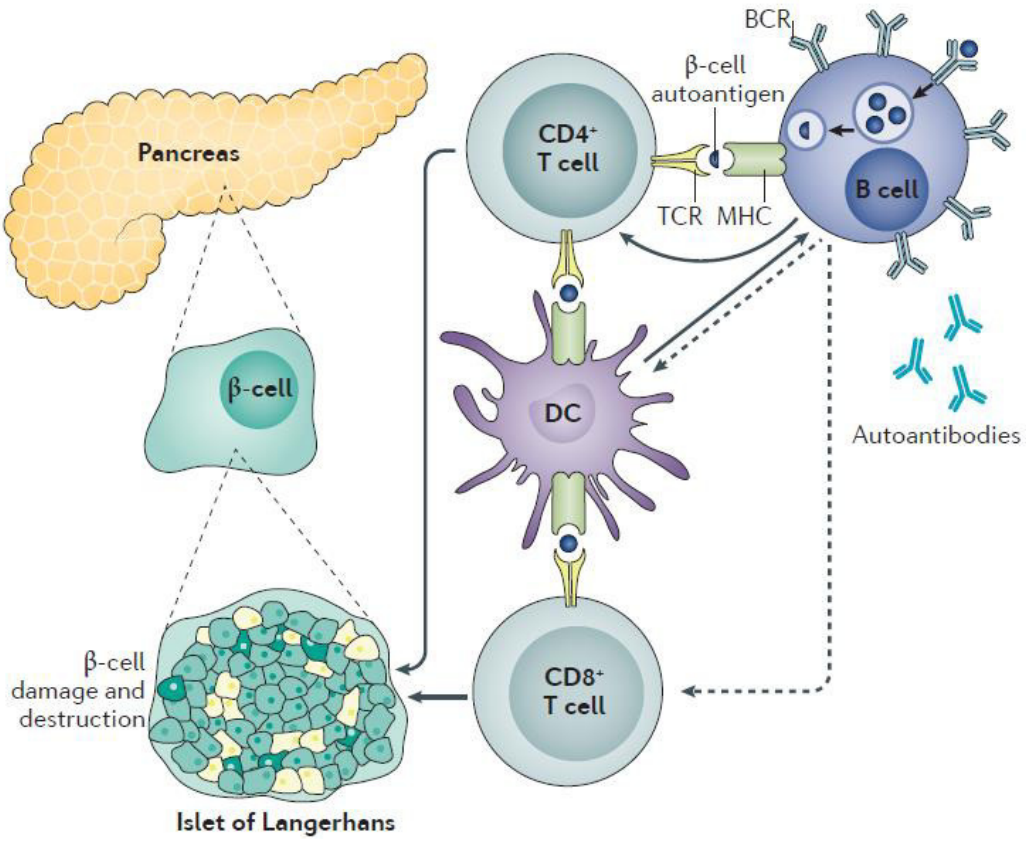

Figure 4. Pathogenesis of T1D. B cells produce antibodies that can recognize and bind to β-cell antigens, marking them for destruction. Additionally, activated B cells interact with dendritic cells (DCs), antigen-presenting cells (APCs) present β-cell antigens to CD4+ and CD8+ T cells, further amplifying the autoimmune response. Autoreactive T cells multiply and release inflammatory molecules and further attract other immune cells, including macrophages and cytotoxic T cells, to the site of inflammation. These immune cells directly attack and destroy the β-cells, the destruction of β-cells leads to a decrease in insulin production, resulting in elevated blood glucose levels (Adapted from [118]).

Along with the progression of the autoimmune response, inflammatory mediators play a decisive role in the pathogenesis of T1D [128], [129]. Pro-inflammatory cytokines such as IL -1β [130], [131], interleukin-6 (IL -6) [132], [133], and TNF-α [134]–[136] further contribute to β-cell destruction. These cytokines facilitate immune cell infiltration, activate antigen-

presenting cells, and promote the autoimmune response within pancreatic islets [129], [137], [138]. At the end, the loss of functional β-cells results in inadequate insulin production, leading to hyperglycemia and the clinical manifestation of T1D.

2.5 Risk factors

The incidence of T1D has increased rapidly in specific regions and shows large differences among different ethnic groups [139]. The overall age-adjusted incidence of T1D varies from 0.1/100 000 per year in the Zunyi region within China to more than 40/100 000 per year in Finland [140], [141]. However, there are also data showing that the north-south gradient of disease incidence is not as strong as previously thought [139], [142], [143]. The two areas with the highest incidence rates, Finland and Sardinia, are 3,000 kilometers apart, while the incidence rate in Estonia, which borders Finland, is only about a quarter of it [140]. Although evidence suggests that such differences in disease incidence follow ethnic and racial distributions, from this it can be seen that other factors have to contribute to the onset of T1D [144]–[146].

2.5.1 Genetic Predisposition

Type 1 diabetes has a strong genetic component, multiple genes have been implicated in disease susceptibility, especially the major histocompatibility complex (MHC, also called the human leukocyte antigen: HLA) class II which is located on chromosome 6p21 being the most influential and responsible for more than half of familiar inherited T1D [147]–[149]. Not all HLA gene mutations increase the same levels of risk of developing T1D. For example, variation of the HLA class II genes (e.g., HLA-DR, HLA-DQ), increase the risk for T1D. Certain HLA alleles, such as DRB1*04:01-DQA1*03:01-DQB1*03:01, are weakly or moderately associated with increased risk, while DRB1*15:01-DQA1*01:02-DQB1*06:02 provides a strong protection [150], [151] (Table 1).

In addition, HLA class I alleles have also been found to be associated with T1D [152]–[155]. HLA-A*0201 is one of the most common class I alleles, occurring at a frequency of >60% in T1D patients [156], [157]. In addition, HLA-A*02 increases the risk in individuals with the high-risk class II DR3/4-DQ8 haplotype [158], [159]. The relationship between susceptible HLA class I alleles and age-related onset and severity of diabetes becomes clear in a non-obese diabetic (NOD) study. Marron *et al.* have generated NOD mice which express human HLA-A*02 and show the A2-restricted T cell responses against pancreatic β-cells s, leading to

earlier onset of T1D, demonstrating the role of HLA I in the development of T1D, at least in mice [160].

In addition, the mutation of a single gene [161], [162], i.e. IDDM2 locus on chromosome 11 containing the insulin gene region [163]–[165], PTPN22 which encodes the lymphoid protein tyrosine phosphatase (LYP) [166], [167], or interleukin (IL)-2 receptor-α gene (IL2RA) [168]–[170], is related to the predisposition to T1D. These genetic factors influence immune response regulation, antigen presentation, and immune cell activation, ultimately contributing to the autoimmune destruction of β-cells.

Table 1. Type 1 diabetes risk associated with HLA-DR and HLA-DQ haplotypes.From [4].

Risk	**HLA**		
	HLA DRB1	HLA DQA1	HLA DQB1
High risk	0401, 0402, 0405	0301	0302
	0301	0501	0201
Moderate risk	0801	0401	0402
	0101	0101	0501
	0901	0301	0303
Weak or moderate	0401	0301	0301
protection	0403	0301	0302
	0701	0201	0201
	1101	0501	0301
Strong protection	1501	0102	0602
	1401	0101	0503
	0701	0201	0303

2.5.2 Environmental factors

Although environmental factors have not been found to directly turn on diabetes onset, it cannot be denied that they act as modulators in the pathogenesis of the disease. Genetic predisposition provides the basis for T1D, and environmental factors are thought to trigger its onset in genetically susceptible individuals [171], [172].

Among the relevant triggers, viral factors are considered to be the main environmental factors that trigger autoimmunity [173]–[176]. Especially enteroviruses are the major viral candidates for causing T1D [177]–[179].

Since 1969, an association between enterovirus infection and the seasonal incidence of T1D has been reported [180], [181]. Hyoty *et al.* found higher titers of enterovirus antibodies in pregnant women whose children later developed T1D [182], [183]. Elshebani *et al.* isolated certain enterovirus strains from T1D patients which affect β-cell function and destruction *in vitro* [184]. Enteroviruses, i.e. coxsackievirus B and rotavirus can either directly or indirectly induce autoimmune responses, leading to inflammation and impaired β-cell function [185]–[189]. Release of type I interferon (IFN-1) and activation of lymphocytes following viral infection has also been observed, thereby accelerating β-cell death and autoimmune processes [190], [191].

Large epidemiological studies have shown that infection with influenza virus [192]–[195], herpesvirus [196], [197], measles, mumps, and rubella can activate immune cells and leading to the destruction of β-cells and insulin deficiency, and diabetes [198]–[202].

Vitamin D can inhibit immune activation and dendritic cell differentiation. Littorin *et al.* observed that the metabolic levels of vitamin D in plasma are low during disease onset in T1D patients [203]. Mathieu *et al.* found increased vitamin D intake associated with reduced incidence in mice [204]. However, studies to prevent T1D progression in susceptible children have all failed [205], [206]. Gut bacterial composition has long been recognized as an important variable affecting the development of autoimmunity and T1D. Whether the interference of antibiotics or faecal transplants aggravates or prevents the occurrence of diabetes has become a hot topic for clinical [207]–[209]. Also gluten intake or cow's milk, which are daily sources of nutrition, were thought to promote islet autoimmunity, as cross-reactivity was found between serum antibodies to albumin and the β-cell surface protein ICA-1 (p69) [210] but interventions could not prevent the T1D prevalence in clinical studies [211].

2.6 Coxsackievirus-associated T1D

Viral infection, especially through enteroviruses as trigger for T1D has attracted much attention. Since 1969 [180], when a significant positive correlation was reported between the total number of new T1D cases and the annual prevalence of coxsackievirus type B4, scientists have not given up digging for the "root" of truth. Moreover, the debate of the potential link between viral infections and the onset or progression of T1D is still going on, despite a large recent meta-analysis from Maria Craig's lab [212].

Coxsackievirus got its name due to its first discovery in 1948 by Dalldorf in the town of Coxsackie, New York [213], [214]. A group of nonenveloped, linear, positive-sense single-stranded RNA viruses belongs to the family Picornaviridae and the genus Enterovirus [215]. The viral capsid consists of four different viral structural proteins; viral proteins 1 to 4 (VP1 to VP4), [216]. Coxsackieviruses mainly contain two types, group A and group B. Group A is divided into 23 serotypes (1-22, 24) according to their antigenic properties, while group B contains only serotypes 1-6 [217], [218]. Coxsackievirus is a common virus that is endemic worldwide usually causes Hand, Foot, and Mouth Disease (HFMD) [219], [220], Herpangina [221], [222], Pleurodynia [223], Viral Meningitis [224], myocarditis [225], [226] and pericarditis [227], patients primarily get infected through contact with respiratory secretions, faeces, or contaminated surfaces.

Enterovirus strains were isolated from pancreatic tissue of patients with onset T1D, which assume that the coxsackievirus could directly infect β-cells, leading to their dysfunction and destruction [184], [228]. Furthermore, CVB-induced β-cells damage involves multiple mechanisms, including viral replication within β-cells, induction of apoptosis and disruption of intracellular signaling pathways, these processes lead to β-cell death and subsequent release of autoantigens, triggering an autoimmune response [229]–[233].

2.6.1 The host immune response

Coxsackieviruses can directly lyse infected cells, and trigger the host immune responses [234], initiated through the activation of viral pathogen-associated molecular patterns (PAMPs) [235] and pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) on the cell membrane surface [236]–[238], cytoplasmic receptors, including RIG-like receptors (RLRs), nucleotide-binding domain-leucine-rich repeat-containing molecules (NLR) and RNA-activated protein kinase R (PKR) [239]–[241].

This leads to the production of pro-inflammatory chemokines and cytokines, such as interferon-gamma (IFN-γ), TNF-α, and IL-1β, which -on the one hand- contribute to the

activation of immune cells and amplification of its own inflammatory response, but -on the other hand- promote β-cell destruction [238], [242]–[246].

Dysregulated, accelerated cytokine production induced by viral infection disrupt immune regulatory mechanisms, leading to the inadequate suppression of autoreactive immune cells and the progression of cell damage [245], [246], as seen in numerous ways also in the 2019 SARS-CoV2 pandemic through the “cytokine storm” [247].

2.6.2 Persistent viral infection

In addition to acute viral infection, low concentrations of CVB can lead to persistent viral infection in pancreatic tissue. Persistent infection is characterized by continued production and replication of viral proteins, which can trigger a chronic inflammatory response, without cell lysis, but impairment and disruption of β-cell function. Hyöty *et al.* found that persistent infection might lead to prolonged activation of dendritic cells to continuously present viral and self-antigens to T lymphocytes, thereby inducing antiviral and autoimmune responses that secondarily trigger β-cell lysis [248]. Richardson *et al.* reported that CVB can persistently infect β-cells, which in turn trigger the synthesis and chronic expression of IFN-α, further stimulates the expression of MHC class 1 molecules and initiates an autoimmune response against β-cells [249], [250]. Stewart *et al.* also confirmed that IFN-α expressed by β-cells in transgenic mice can lead to insulitis and islet cell destruction by activating T cells [251].

Multiple persistent virus infections will stimulate the response of virus-specific Cytotoxic T lymphocytes (CTLs) in the lymphoid tissue of the digestive tract (the main replication site), and these persistently infected β-cells are likely to be the main targets of virus-specific CTLs [248].

2.6.3 Molecular mimicry

Molecular mimicry occurs when similarities between foreign and self-peptides favor an activation of autoreactive T or B cells by a foreign-derived antigen in a susceptible individual [252]. When derived molecules from pathogens (such as bacteria, viruses, or other microorganisms) are closely similar to their own molecules in the host organism, they can lead to cross-reactive immune responses [252]. In autoimmune diseases, the immune system cannot distinguish between "self" and "non-self" and thus produces not only antibodies or T cells against foreign pathogens, but also immune cells against self-antigens, and as a result, these immune cells make mistakes Attacks the host's own organ tissues, leading to autoimmune diseases [253]–[256].

There are some T1D-related autoantigens, which provide evidence that β-cell self-antigens have a partial sequence homology with CVB peptides, including tyrosine phosphatases (IA-2 and IAR), GAD65 and heat shock protein 60/65 (HSP60/65) [257], [258]. The IA-2/IAR diabetogenic peptide, which is a significant self-antigen targeted in T1D, can be detected by antisera induced by CVB4. This indicates that there is a shared recognition between the antibodies and T cells raised against CVB peptides and the self-antigens present in islets [259].

Consequently, this prompts the activation of autoreactive T cells that target both the viral epitopes and the β-cell antigens. This cross-reactivity can lead to the immune system identifying and attacking β-cells s, causing their destruction (Figure 5). Additionally, CVB infection may alter the antigen-presenting capabilities of immune cells, promoting the presentation of β-cell autoantigens to autoreactive T cells, thereby amplifying the autoimmune response.

2.6.4 Bystander activation

When inflammation occurs due to viral infection, immune cells such as macrophages and dendritic cells were activated to engulf foreign antigens and even self-antigens from damaged tissues [260], [261]. The antigens are then combined with MHC molecules and presented on the surface of APCs later activate the adaptive immune response (Figure 5). In the presence of infection or inflammation, the local microenvironment may become pro-inflammatory, leading to the secretion of various cytokines and chemokines [262], [263]. These soluble factors can produce a "bystander effect," meaning that inflammation and immune activation are not strictly localized to the site of infection or injury [264]. Inflammatory signals can spread and reach other tissues and lymphoid organs and affect the activated T cells and B cells with autoantigen-specific receptors [265].

In this bystander effect, the activation of autoreactive immune cells may lead to the recognition of self-antigens in healthy tissue as foreign antigens. As a result, activated autoreactive immune cells may attack healthy tissue, leading to autoimmune responses and the development of autoimmune diseases.

Both mechanisms of molecular mimicry and bystander activation could explain the association between enterovirus infection and T1D pathogenesis. Horwitz *et al.* demonstrated that induction of T1D by CVB was more likely to be triggered by bystander activation instead of molecular mimicry [266]. Interestingly, mice with a susceptible MHC-I able to recognize either autoantigens or coxsackievirus epitope did not develop disease after viral infection. In contrast,

mice with resting auto-reactive T cells that recognized islet antigens but did not cross-react with the virus developed T1D after viral infection [260]. From this, it can be seen that T1D induction by virus was the result of auto-reactive T-cell re-stimulation by the pro-inflammatory milieu induced by infection.

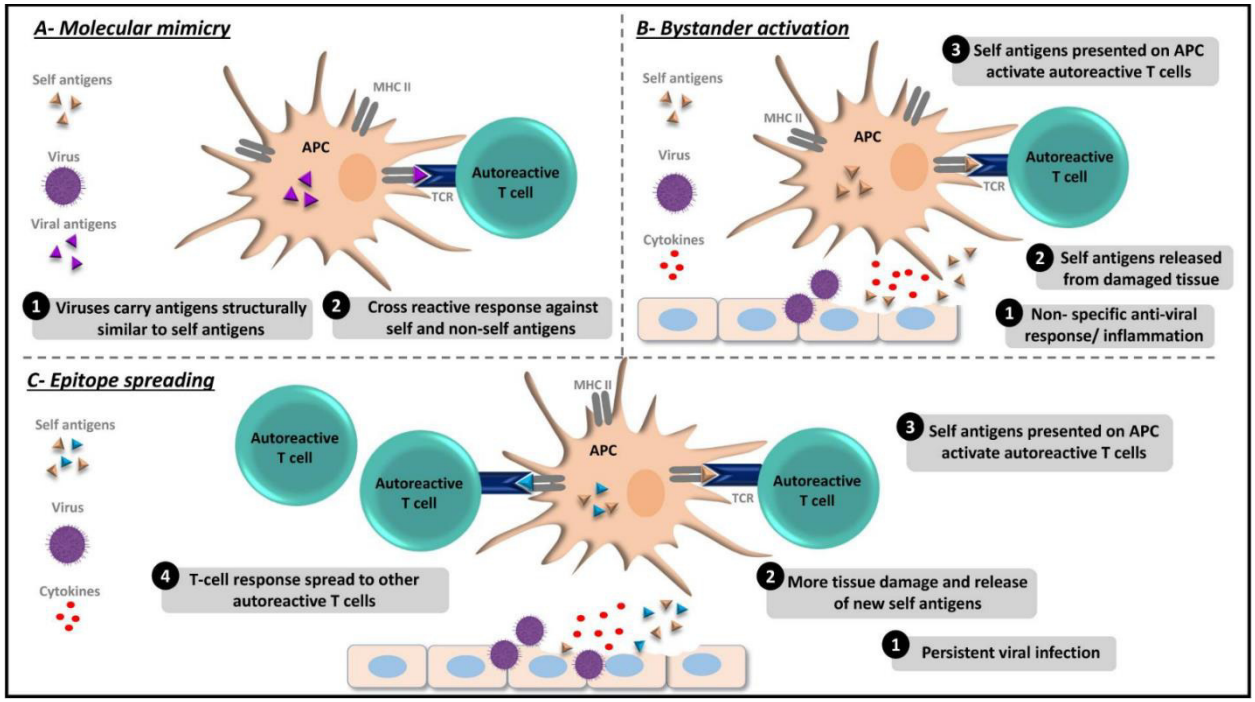

Fig.5. Mechanisms of virus-induced autoimmunity. (A) Molecular mimicry: Viral antigens are extremely similar to the host's own antigens, lead to a cross-reactive immune response, and the immune system cannot distinguish "self" from "non-self", causing immune cells to mistakenly attack the host's own healthy cells or organs, resulting in autoimmunity disease. **(B) Bystander activation:** In this situation, non-specific antiviral immune response leads to the emergence of a localized pro-inflammatory environment as well as the production of self-antigens and inflammatory cytokines from the affected tissue. Later, APCs take up and display these self-antigens, leading to the activation of previously inactive but autoreactive T cells in the surrounding area. Therefore, autoimmunity is triggered, causing the immune system to target its own tissues. **(C) Epitope spreading:** Persistent viral infection leads to persistent tissue damage and continues self-antigens producing. APCs taken up and presenting self-antigens which results in autoreactive T cells activation. As the immune response continues, some immune cells can recognize and respond to the newly exposed self-antigens. Over time, the immune response expands to target multiple epitopes on the same or different proteins. (Adapted from [267]).

Mechanisms governing the survival and growth of host cells could potentially offer a favorable environment for viral replication mechanisms. One prime example of a classical pathway responsible for managing organ dimensions, tissue stability, and cell survival is the Hippo signaling pathway. This pathway is intricately connected to the underlying causes of cancer and metabolic disorders [268], [269], and recently also to hosts' viral responses [270]. Therefore, we started a large investigation on the Hippo Pathway in the regulation of viral infection immune response and β-cell death.

2.7 The Hippo Pathway

The Hippo signaling pathway is a remarkable regulatory mechanism that controls cell growth and proliferation, tissue development and homeostasis and organ size [271]–[273]. It was discovered in the early 2000s in the fruit fly Drosophila melanogaster and thus named after the Hippo gene identified in Drosophila. This signaling pathway acts as a "brake" to limit cell growth and prevent tumor formation. The key components of the Hippo signaling pathway include: (a) MST1/2 (mammalian STE-like 1 and 2), (b) LATS1/2 (large tumor suppressor 1 and 2) and (c) YAP /TAZ (Yes-associated protein and transcriptional coactivator with PDZ-binding motif).

MST1/2. These serine/threonine kinases serve as core upstream components of the Hippo pathway and initiate the signaling cascade. MST1/2 kinases are activated by various upstream signals, such as cell density, mechanical stress, and cell polarity. And regulate apoptotic signaling through a variety of phosphorylation-dependent substrates. MST1/2 modulates the transcriptional regulation and protein stability of many substrates.

LATS1/2. These kinases phosphorylate downstream effectors, including YAP and TAZ, marking them for degradation, sequestering them in the cytoplasm and preventing their translocation to the nucleus.

YAP and TAZ are transcriptional co-activators and key effectors of the Hippo pathway.

When not phosphorylated, YAP/TAZ reside in the nucleus where they interact with transcription factors, including TEAD family, and promote gene expression associated with cell proliferation and tissue growth.

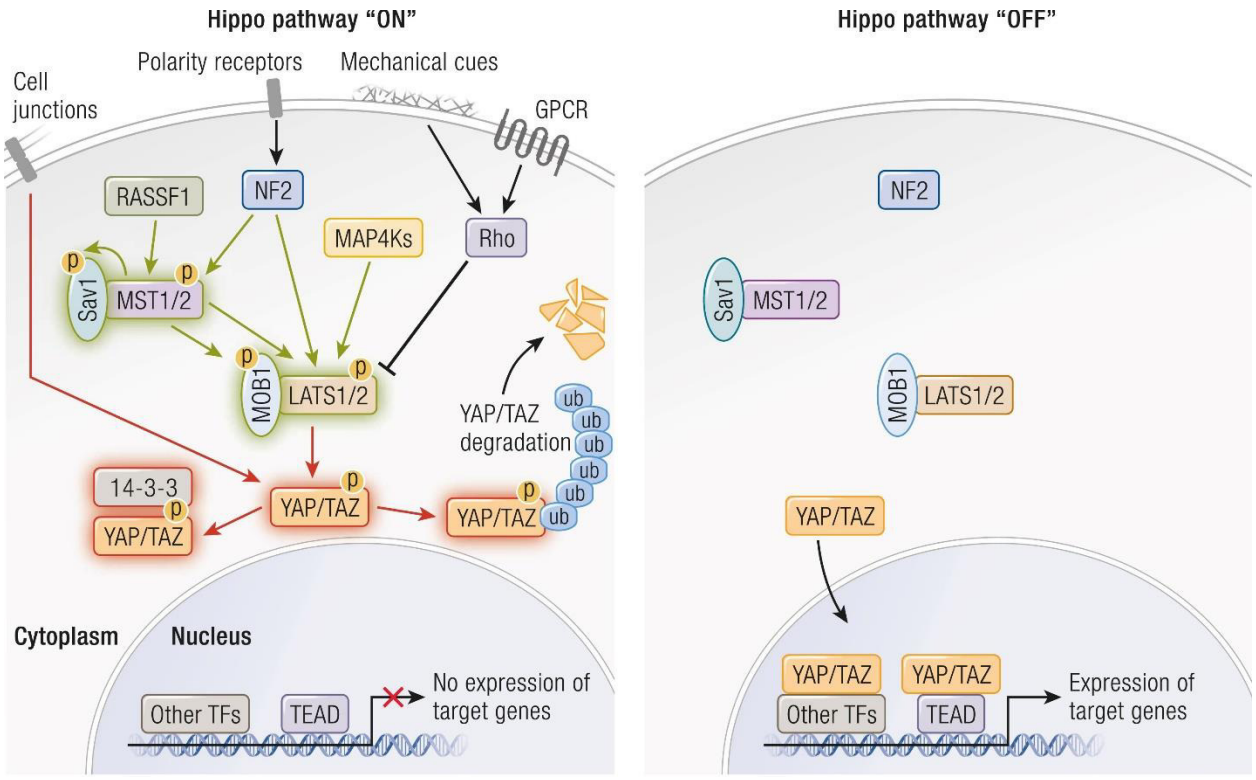

Figure 6 The mammalian Hippo pathway. In mammals, when the Hippo pathway is ON, activated MST1/2 and Salvador homolog 1 (SAV1) phosphorylate and activate the LATS1/2. Then the adaptor proteins MOB kinase activators 1 (MOB1) binds and activates LATS1/2 kinase. Activated LAST1/2 together with MOB1 subsequently phosphorylate and inactivate transcriptional co-activators YAP/TAZ, which leads to YAP/TAZ cytoplasmic retention (inactive) and degradation by their interaction with 14-3-3 proteins. In contrast, When the Hippo pathway is OFF, the kinases MST1/2 and LATS1/2 are inactivated, the YAP/TAZ can translocate to the nucleus and interact with TEAD transcription factors and induces the expression of target genes. (Adapted from [274]).

2.7.1 Yes-associated protein 1 (YAP1)

In 1995, Sudol *et al.* cloned and characterized a protein that binds to the Src homology domain 3 (SH3) of the yes proto-oncogene product and named it YAP1, also known as YAP or YAP65 [275]. In mammals, YAP1-1 and YAP1-2 are the two subtypes of YAP1 gene product (Figure 7); YAP1-2 has an extra 38 amino acids encoding the WW domain compared with YAP1-1[275], [276]. What they have in common is a proline-rich region at the N-terminus of the YAP1 protein followed by the TEAD transcription factor interacting domain (TID), which serves to recruit and bind transcriptional coactivators and to regulate target gene expression[277], [278]. The end of the WW domain is connected to Src Homology 3 binding motif (SH3-BM), following the SH3-BM is a transcription activation domain (TAD) and a PDZ domain-binding

motif (PDZ-BM), which is responsible to bind and initiate the transcriptional expression of specific genes (Figure 7).

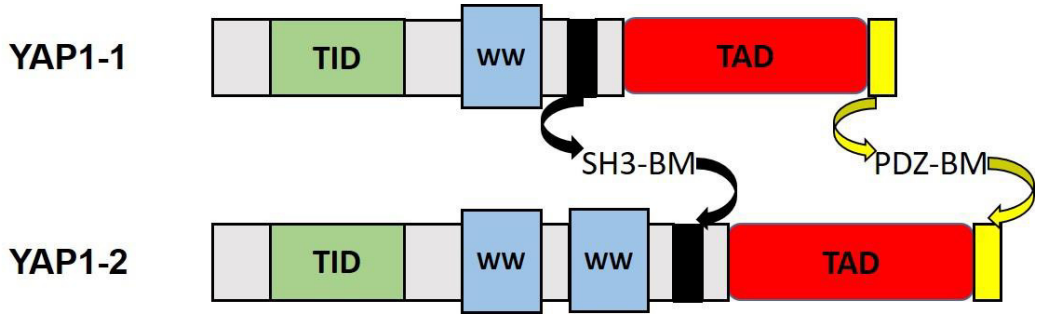

Figure 7. Modular Structure of YAP1 Isoforms. Two splice isoforms of the YAP1 gene product are YAP1-1 and YAP1-2, the latter has more WW domain, both contain TID, SH3-BM and PDZ-BM. (Adapted from [279]). Created with BioRender.com

YAP1 is a transcriptional co-activator of the Hippo signaling pathway to regulate self-renewal [280], tissue regeneration [281]and organ size[282] and apoptosis [283]–[287]. When YAP translocate to the nucleus, it forms a complex with TEAD proteins and binds to specific DNA sequences in the promoters of target genes. This YAP-TEAD complex enhances the transcriptional activity of TEAD and promotes the expression of genes. [240]– [244]

A large number of clinical studies have shown that YAP promotes cancer development by inhibiting apoptosis. YAP is overexpressed in tumors, which leads to accelerated tumor growth, such as lung tumors [288], [289], pancreatic tumors [290], [291] and colorectal cancer [292], [293]. In addition, YAP can increase the expression of anti-apoptotic genes, like Cyclooxygenase-2 (COX-2) [294], BIRC 5 (Survivin) [295], [296], glucose-transporter 1 (Glut1) [297] by interacting with TEAD 1-4. Additionally, YAP can inhibit apoptosis by increasing glycolysis [297], [298] or enhancing the autophagic Flux [299]–[302]. Transcription factor AP-2 Gamma (TFAP2C) [303], [304], WW domain binding protein 5 (WBP5) [305], [306], lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) [307], serine/threonine kinase 38 like (STK38L) [308], cyclic adenosine monophosphate (cAMP) response element-binding (CREB) [309], forkhead box protein A1 (FOXA1) [310], [311] and S100 calcium-binding protein A1 (S100 A1) [312]*et al.* have been reported as activators of YAP which impede apoptosis.

On the other hand, clinical studies have also shown that YAP may have the function of slowing down cancer development by promoting apoptosis [313]–[316]. Cottinin *et al.* demonstrated that YAP could trigger DNA damage-induced apoptosis in hematological cancer [317]. Yuan M *et al.* illustrated that YAP is a tumor suppressor in breast cancer [318]. YAP can interact

with a tumor suppressor p73 to promote pro-apoptotic genes expression, such as p53AIP1 [313], Bax [314], [319], DR5 [315], and PUMA [316]. Ras association domain family 1 isoform A (RASSF1A) suggested as a tumor suppressor allows YAP to move to the nucleus and to interact with p73, the YAP-p73 complex results in transcription of the pro-apoptotic target gene PUMA in breast cancer cells and Ankyrin Repeat Domain 1 (ANKRD1) [316], [320], [321]. Early growth response-1 (EGR-1) is a nuclear protein, which interacts with YAP to further induce cell death [322]–[324]. G-protein-coupled receptors (GPCRs) [325], [326], protein kinase A (PKA) [327], Ras association domain family member 6 (RASSF6) [328], crumbs-3 (CRB-3) [329]–[331] and AMP-activation protein kinase (AMPK) [332], [333] can induce apoptosis via inhibition of YAP.

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

Mammalian sterile 20-like kinases (MST kinases) are a group of serine/threonine kinases of the sterile 20 (STE20) family. In 1995, Creasy C *et al.* cloned and characterized a human protein kinase which is Homology to the yeast kinase Ste20, and named them MST1 (also known as Stk4 and Krs2) and MST2 (also known as Stk3 and Krs1) [334]–[336]. Human MST1/2 are highly homologous, approximately 75%. They have a unified multi-domain structure (Figure 8), including an N-terminal kinase domain, a central catalytic domain, which contains all the necessary elements for ATP binding and substrate phosphorylation, and a C-terminal regulatory region. C-terminal regulatory region further divided into an autoinhibitory domain, which interacts with the catalytic domain to control the kinase activity of MST1/2 and a coiled-coil dimerization domain, also known as Salvador-RASSF-Hippo (SARAH) domain, which mediates homodimerization or heterodimerization with other SARAH-containing proteins, such as SAV1 and RASSF proteins. The caspase cleavage sites located between the kinase domain and the autoinhibitory domain [337].

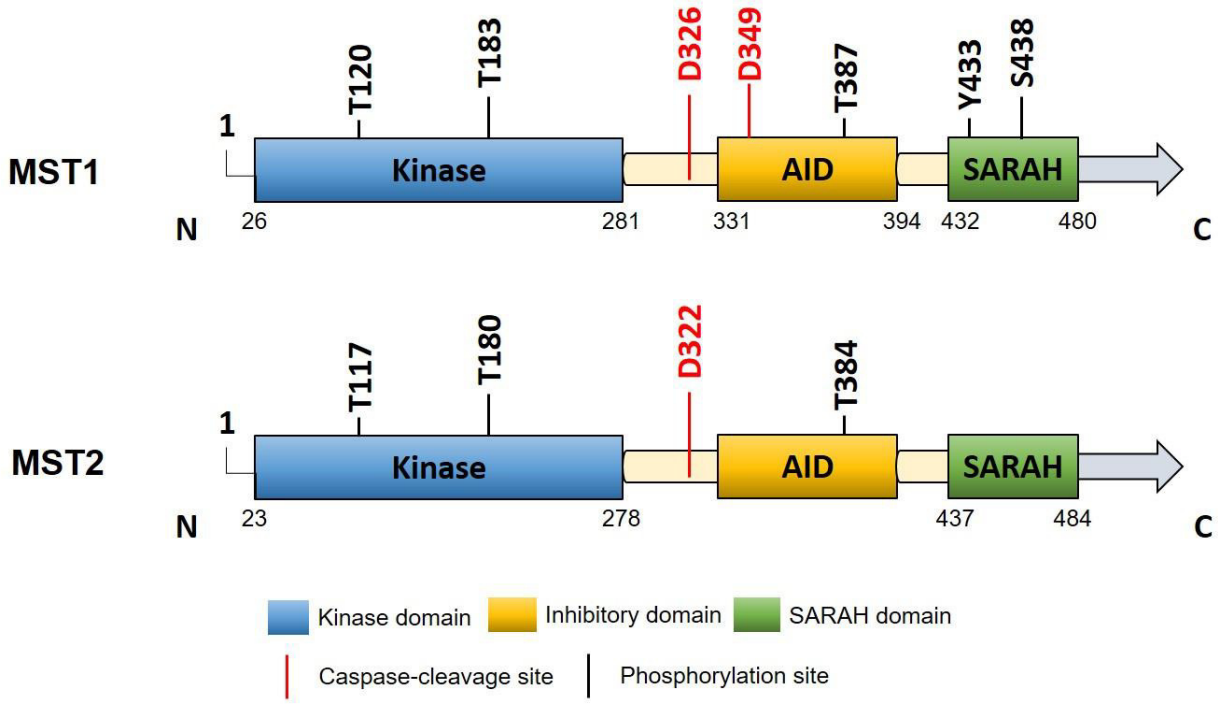

Figure 8. The structure of MST1/2 kinases. Highly homologous, MST1/2 have the same organizational structure over species, including an N-terminal kinase domain, a C-terminal SARAH domain and an autoinhibitory domain (AID). MST1 and MST2 have different caspase cleavage sites between the kinase domain and AID, as well as different phosphorylation sites. (Adapted from [337])Created with BioRender.com

MST1/2 are the key components of the Hippo pathway. Activation of MST1/2 leads to the phosphorylation and activation of downstream LATS1/2, resulting in the inhibition of the transcriptional coactivators YAP/TAZ. By suppressing YAP/TAZ activity, MST1/2 helps prevent uncontrolled cell growth and tumor formation [338]. Numerous clinical data show that MST1/2 expression or activity is frequently reduced or inhibited in various types of cancer, this suggests that MST1/2 may be involved in tumorigenesis and progression as a tumor suppressor [339], [340]. Moreover, MST1/2 can induce programmed cell death in response to various stress signal, they activate downstream effectors, such as the MOB kinase activator (MOB) [341], [342]. As the cellular process responsible for recycling damaged or unnecessary cellular components, MST1/2 activate autophagy in response to cellular stress and facilitate the degradation of unwanted cellular material [343]–[345]. This also contributes to the immune defense against infections. MST1/2 also play a crucial role in immune response, they activate T cell receptor (TCR) signaling and control T cell activation and proliferation. MST1/2 mediate

the rearrangement of the actin cytoskeleton, which is critical for the formation of the immunological synapse and the proper activation of T cells [346]–[349]. MST1/2 can also modulate the activation of macrophages, influencing the production of cytokines and other immune mediators [350].

In addition, MST1/2 plays an important role in cerebrovascular disease [351], [352], neurodegenerative disorders [353], [354], [355], neuromuscular disorders [356], [357] and cerebral tumor [358], [359].

2.7.3 The Hippo Pathway's influence on β-cell death

β-cells survival and function play a decisive role in the development of type 1 and type 2 diabetes. Recent studies have unveiled that the key regulators of Hippo pathway, such as MST1, LAST1/2 and Merlin/NF2 and YAP are involved in maintaining β-cell survival and function [271], [360]–[363].

The Hippo pathway's activation seems to be a key defense mechanism against stressors that threaten β-cell viability, such as oxidative stress, ER stress, and cytokine-induced apoptosis. The Hippo pathway interacts with multiple signaling pathways known to affect β-cell function and survival. Its exhibits crosstalk with the insulin-signaling pathway, which is critical for β-cell survival and insulin secretion. Disruption of this crosstalk may lead to impaired β-cell survival and function, ultimately leading to the development of diabetes. Ardestani *et al.* identified that the MST1 phosphorylates PDX1 at T11 site leading to its ubiquitination and degradation thereby causing defective β-cell function in rodent and human islets under diabetic conditions [360]. In contrast, genetic inhibition of MST1 could rescue the β-cells from apoptosis and restore β-cell function under diabetic conditions [360]. In line, LATS2 overactivation induces β-cell failure by increasing β-cell apoptosis and impairing β-cell function. Overexpression of LATS2, acting downstream of the MST1 kinase, was shown to trigger apoptosis and impair β-cell function [274]. Furthermore, Yuan *et al.* demonstrated Merlin as an upstream component of the Hippo pathway, which also regulates β-cell survival. Depletion of Merlin protects β-cells from apoptosis under diabetic conditions [361], [363].

For both T1D and T2D, manipulating the Hippo pathway components offers promising therapeutic strategies to protect β-cells and preserve insulin production. Uncovering the complex link between the Hippo pathway and β-cell death has the potential to revolutionize our understanding of diabetes pathogenesis.

2.7.4 The Hippo Pathway's Response to Viral Infection

As a regulator of homeostasis, the Hippo pathway interacts with antiviral response [270]. Activation of PRRs by viral pathogens stimulates the Hippo pathway, regulating the production of type I interferons and pro-inflammatory cytokines [364]. This activation helps to mount an effective antiviral immune response and control viral replication, while protecting the host cell from accelerated inflammation (Figure 9). Interferon Regulatory Factors (IRFs) are a family of transcription factors that play a crucial role in the regulation of antiviral responses and immune activation [365], [366]. Wang *et al.* demonstrated that YAP could directly inhibit IRF3 function, thereby reducing IFN-β production and the innate antiviral response to viruses. While, studies show YAP/TAZ can interact with IRFs, influencing their transcriptional activity and downstream gene expression. YAP/TAZ have been shown to cooperate with IRF3 and IRF7 to enhance the production of type I interferons, key molecules in antiviral defense [366]. Moreover, Jiao shows that the expression of IRF3 in gastric cancer was positively correlated with the expression of YAP and its target genes, demonstrating that IRF3 acts as an agonist of YAP [367].

Following viral infection, YAP-deficient mice exhibit enhanced innate immunity and reduced viral load [368]. Viral infection causes IKKε to phosphorylate YAP Ser403, which in turn triggers its lysosomal degradation, thereby enhancing cellular antiviral responses [368]. YAP/TAZ has been shown that interact with TLR signaling pathways, regulating the expression of immune-related genes. YAP/TAZ activation can enhance TLR-mediated cytokine production, such as IL-6 and TNF-α, thereby influencing the magnitude of the inflammatory response [369]–[371].

Viral infection can trigger activation of the Hippo pathway in various cell types, including immune cells. When activated, the Hippo pathway regulates the transcriptional activity of YAP/TAZ, which regulates the expression of immune-related genes, thereby affecting the outcome of viral infection. Zhang *et al.* have shown that YAP/TAZ can inactivate virus-induced TBK1-IKKε by inhibiting ubiquitination of Lys63 in TBK1. Expression of YAP/TAZ impairs virus-driven viral resistance and restores viral replication.

Meng F *et al.* show that MST1 inhibits virus-induced TBK1/IKKε activity and restores virus replication [365]. Likewise, TLR signaling that can interact with MST1 to modulate host immune responses and promote bacterial killing has been demonstrated by White *et al.* [370].

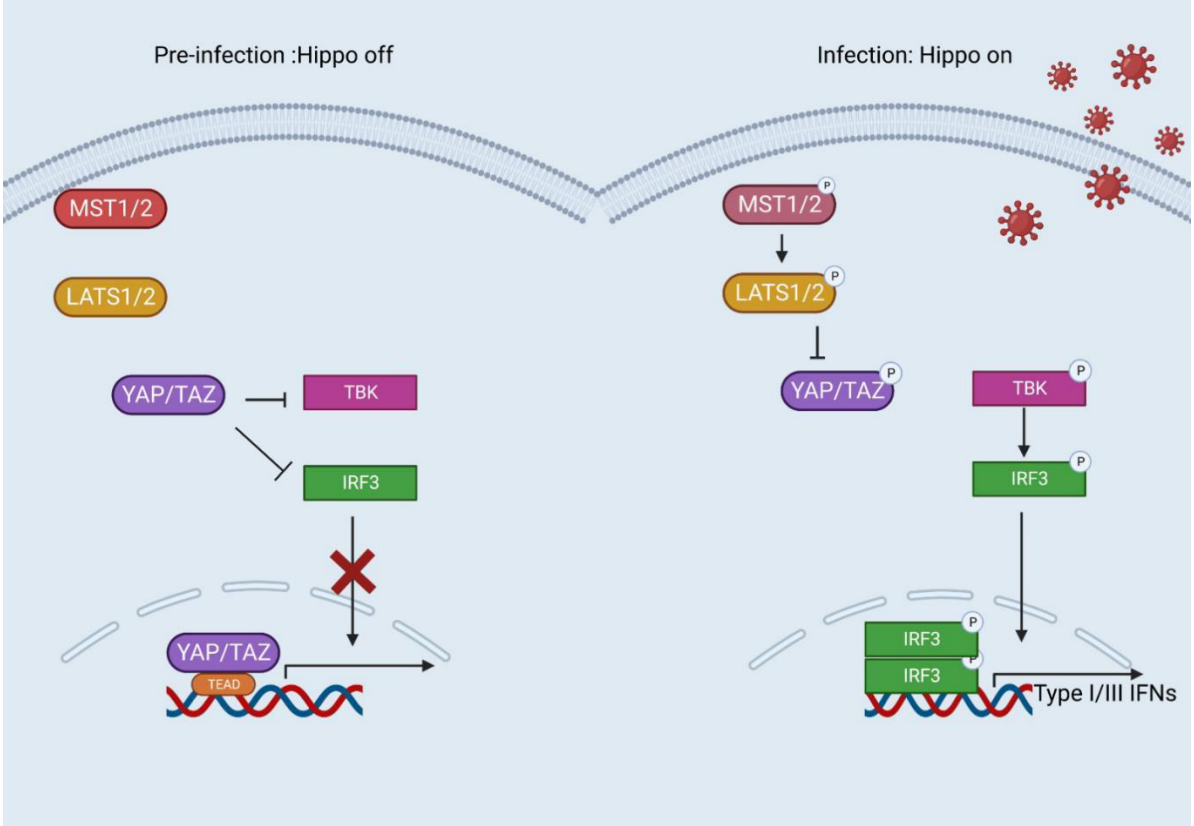

Figure 9. Hippo-YAP interact with TBK1 regulate viral infection. Hippo pathway off, YAP can either inhibit TBK1, further inhibit IRF3 or YAP directly inhibit IRF3, there no cytokine and chemokine release from IRF3. Hippo pathway on, activated MST1/2 phosphorylated YAP, then TBK1 stimulate IRF3 release anti-inflammatory factors, which against viral infections [270]. Created with BioRender.com

Altogether, YAP can either enhance or decrease virus-induced responses. Regulate YAP or other key factors in the Hippo pathway to protect the function, mass and regeneration of β-cells is the top priority of our research. Understanding the crosstalk between YAP and innate immune signaling pathways provides insights into the regulatory mechanisms of immune responses. Targeting specific points of crosstalk between YAP and innate immune signaling pathways may offer therapeutic opportunities for modulating immune responses and treating immune-mediated diseases.

2.8 Aim of the thesis:

Enteroviruses and their specifical contribution to the development of T1D have been a long and controversial story, and whether there is a virus-association in T2D is a mystery and has not been adequately addressed. Insulin therapy is currently the only effective treatment for T1D and oral antidiabetics, which improve insulin sensitivity and secretion for T2D, all those are only treatments and not a cure [372]. Therefore, we must look for new opportunities to cure diabetes by understanding the pathogenic mechanisms.

My specific aims were:

Part 1: Identify and localize enterovirus RNA presenting the pancreas in T2D donors.

(1) Are enteroviral RNAs in the endocrine or exocrine pancreas of organ donors with T2D?

(2) Are enterovires associated with islet lymphocyte infiltration, islet inflammation, and β-cell loss in T2D?

Part 2: The Hippo pathway effector YAP in viral infection in the T1D pancreas: protector or accelerator?

(1) Is YAP expressed in the pancreas of T1D and AAb+ organ donors and does it colocalize or correlate with enteroviral RNA expression in the pancreas?

(2) Does YAP influence CVB replication, islet inflammation and β-cell apoptosis?

(3) Does YAP modulation affect the viral response in islets?

(4) Is there a Hippo pathway feedback loop that coordinates YAP effects and virus pathophysiology?

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3 Results

Manuscript I

Enteroviral infections are not associated with Type 2 diabetes

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My Contribution:

Performed experiments, analysed data, drafted the paper and assembled the figures for: Figure 1 (A-I partially), S-Figure 1(partially), S-Figure 1(A, D), S-Table 1 and 2 (partially), Contributed in editing the paper.

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Enteroviral infections are not associated with type 2 diabetes

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Introduction: For more than a century, enteroviral infections have been associated with autoimmunity and type 1 diabetes (T1D). Uncontrolled viral response pathways repeatedly presented during childhood highly correlate with autoimmunity and T1D. Virus responses evoke chemokines and cytokines, the "cytokine storm" circulating through the body and attack cells especially vulnerable to inflammatory destruction. Intra-islet inflammation is a major trigger of β-cell failure in both T1D and T2D. The genetic contribution of islet inflammation pathways is apparent in T1D, with several mutations in the interferon system. In contrast, in T2D, gene mutations are related to glucose homeostasis in β cells and insulin-target tissue and rarely within viral response pathways. Therefore, the current study evaluated whether enteroviral RNA can be found in the pancreas from organ donors with T2D and its association with disease progression.

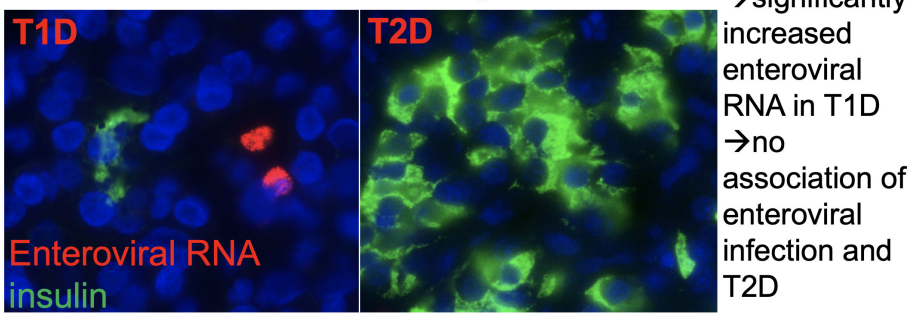
Methods: Pancreases from well-characterized 29 organ donors with T2D and 15 age- and BMI-matched controls were obtained from the network for pancreatic organ donors with diabetes and were analyzed in duplicates. Single-molecule fluorescence *in-situ* hybridization analyses were performed using three probe sets to detect positive-strand enteroviral RNA; pancreas sections were co-stained by classical immunostaining for insulin and CD45.

Results: There was no difference in the presence or localization of enteroviral RNA in control nondiabetic and T2D pancreases; viral infiltration showed large heterogeneity in both groups ranging from 0 to 94 virus$^+$ cells scattered throughout the pancreas, most of them in the exocrine pancreas. Very rarely, a single virus$^+$ cell was found within islets or co-stained with CD45$^+$ immune cells. Only one single T2D donor presented an exceptionally high number of viruses, similarly as seen previously in T1D, which correlated with a highly reduced number of β cells.

Discussion: No association of enteroviral infection in the pancreas and T2D diabetes could be found. Despite great similarities in inflammatory markers in islets in T1D and T2D, long-term enteroviral infiltration is a distinct pathological feature of T1D-associated autoimmunity and in T1D pancreases.

KEYWORDS

type 2 diabetes, enterovirus, coxsackievirus, islets, pancreas



GRAPHICAL ABSTRACT

Highlights

- Enteroviral RNA$^+$ cells were found scattered throughout the whole pancreas from organ donors.
- They were very rarely seen within islets.
- Enteroviral RNA$^+$ cells were similar in controls and T2D donors.
- CD45$^+$ lymphocytes were increased and β-cell area decreased in the pancreases in T2D.
- In contrast to T1D, enteroviral infiltration is not a pathological feature of T2D pancreases.

Introduction

Ever since Cerasi and Luft have recognized that type 2 diabetes (T2D) is caused by the relative inability of β cells to secrete sufficient amounts of insulin to compensate for insulin resistance, and not by insulin resistance itself (1), underlying mechanisms of this β-cell defect have been searched for and found quite complex, with β-cell inflammation as a major factor in both T1D (2) and T2D (3, 4). Low-grade inflammation is detectable in serum (5) and in single cells within the islets in the pancreas (3, 6) and correlates with accelerated β-cell loss in T2D (7).

Although there is little sign of an acute viral infection in the pancreas, the association of enteroviruses and autoimmune disease, especially T1D (8), has been identified through highly sensitive methods and analyses of carefully collected patients' material in the context of well-powered studies (9–11). The increased presence of enteroviral RNA in the pancreas from organ donors with T1D has just been confirmed in a large meta-analysis (12). Especially, long-duration or multiple enterovirus B (EVB) infections correlate with islet autoimmunity and T1D progression (13, 14). Isolated enteroviruses from human pancreas obtained via biopsy near the T1D onset could be transmitted to cells in culture and produced an immune response (15). Enteroviruses can persist in the pancreas and chronic and/or repeated infection results in the production of inflammatory mediators and triggers an inflammatory response against islet cells (16). The virus is suggested to carry a deletion at the 5′ terminus that renders it persistent and non-cytopathic (17). Using vaccines against coxsackieviruses group B, preclinical studies have successfully prevented infection and CVB-induced diabetes, and clinical studies are in progress (18).

Stark stimulation of viral response pathways seems to foster autoimmune as well as metabolic disease, as seen during the SARS-CoV-2 pandemic, where T1D onset after COVID-19 was identified in several (19, 20) but not all studies (21). More apparent is the highly increased severity of COVID-19 in patients with T2D and obesity in one hand and progression of T2D after COVID-19 on the other (22, 23), with circulating chemokines and cytokines orchestrating a "cytokine storm" that impacts multiple organs in the body. Cells especially vulnerable to inflammatory attack are destroyed and viral response pathways uncontrolled (24) (9, 25),.

Inflammation is a major trigger of β-cell failure, loss of function and apoptosis, both in autoimmune T1D and T2D (4). Islet inflammation as a primary modulator of the progression of T2D had initially been challenged (e.g., reviewed here (26, 27), but has been confirmed by numerous studies from different laboratories and now achieved its acceptance in textbooks (e.g. (28)). Numerous environmental factors such as not only viral infection but also chronic stress, overnutrition which leads to "gluco- and lipotoxicity," islet amyloid and islet amyloid polypeptide (IAPP) toxic oligomer disposition in islets and bacterial LPS alone or in concert lead to islet inflammation (29–34). Also, other mechanisms of β-cell failure in T1D and T2D, such as dedifferentiation and loss of identity, have been suggested to result from inflammatory insults (35, 36).

Significantly higher protein and mRNA levels of cytokines and chemokines such as IL-1β, IL-6, IL-8, IP-10, IL-17, and MCP1 (3, 6, 27, 32, 37, 38) together with macrophage infiltration have been identified in islets; alterations were observed in *in-vitro* and *in-vivo* models of T2D, in isolated islets and in autopsy-pancreases from donors with T2D. These changes of immune components, specific cytokines, and chemokines, and the occurrence of apoptosis, confirm that an inflammatory response is involved in the pathogenesis of T2D (39, 40).

The genetic contribution to islet inflammation pathways is apparent in T1D, with polymorphisms in the interferon system (41, 42) and interferon and viral infection signatures identified in islets of organ donors with T1D (43, 44). Even in T2D, where gene mutations are related to glucose homeostasis in β cells and insulin target tissues (45), genetic variants affecting viral response pathways have been identified; for example, a TYK2 promoter variant associated with a deteriorated cytokine response has been identified as risk factor for T1D as well as T2D (46) and correlates with increased T2D severity (47).

As coxsackievirus infection is associated with β-cell dysfunction and apoptosis (17, 48), a connection with T2D has been hypothesized. The European Prospective Investigation of Cancer-Norfolk study investigated the association between infection, coxsackievirus B serotype 1–5 seropositivity, and T2D, but no correlation between coxsackievirus B neutralizing antibodies and T2D has been found (49). The presence of the enterovirus-specific viral capsid VP1 within islet cells has been found more often in pancreases of patients with T1D than in those with T2D, and only rarely in nondiabetic controls (48, 50). Despite these previous studies, it remained unclear whether there is indeed more enteroviral disposition in the pancreas associated with T2D. We therefore applied a deep and thorough analysis of enteroviral RNA by high-sensitivity single-molecule fluorescence *in-situ* hybridization (FISH), which had been previously demonstrated increased viral RNA in the pancreas of patients with T1D and with islet associated autoimmunity (51), to the network for pancreatic organ donors with diabetes (nPOD) collection of well-characterized pancreases from organ donors with T2D and their age- and BMI-matched controls (52).

Material and methods

Pancreas autopsy material

This study used formalin-fixed paraffin-embedded (FFPE) pancreatic tissue sections obtained from well-characterized organ donors from the nPOD. Donors included 29 with T2D (average disease duration 9 years, range 0.25–26 years, and 15 control donors (without diabetes; Supplementary Table S1). Mean donor age for both groups is 51 years and mean BMI is 28.5 (controls) and 32.9 (T2D). Results were compared to a previous analysis of 15 organ donors with T1D from nPOD and their nondiabetic controls (14; mean donor age 22 years, BMI 24 and 25, respectively) (51).

Virus detection in FFPE tissue samples

Custom Stellaris® FISH Probes against enteroviral RNA labeled with Quasar 570 were used to detect viral RNA as described previously (Biosearch Technologies, Inc., Petaluma, CA, USA) (51, 53). The three probe sets recognize various enteroviral strains for positive-strand enteroviral RNA, CVB_1 was designed on the CVB3 consensus-based sequence (M33854.1), 106 genome sequences of the enterovirus group B family enteroviruses were aligned, and sequences were then divided into three subgroups based on sequence similarities (CVB_1, CVB_2, and CVB_3) (54). The following stepwise previously established highly sensitive protocol (53) was performed for enterovirus mRNA detection by smFISH in pancreatic tissue sections:

Deparaffinization of FFPE tissue sections. Removal of paraffin by a series of Xylene washes (20 min at 70°C; 10 min at 70°C; 10 min at room temperature), followed by rehydration by ethanol (EtOH; 100%, 100%, and 95%) for 10 min each and for 1h in 70% EtOH at room temperature and rehydrating with RNase free water 2 times for 2 min, all under constant steering.

Prehybridization. Incubation with 0.2M HCl for 20 min at room temperature, transfer to a 50-ml tube with prewarmed 2xSSC and incubation at 70°C for 15 min, phosphate-buffered saline (PBS) 2 times for 2 min at room temperature, incubation with 37°C pepsin (Sigma-Aldrich) for 10 min, washing 2 times with PBS for 1 min and with 0.5% Sudan Black (Sigma-Aldrich) in 70% EtOH for 20 min at room temperature to quench remaining autofluorescence, followed by serial washings with PBS and washing buffer (1xSSC,10% formamide).

Hybridization. Three probes were diluted 1:100 in hybridization buffer (10% w/v dextran sulfate, 10% formamide, 2xSSC) and applied to the sections, glass-covered and incubated at 37°C for 12h–14h in a humidified chamber.

Post-hybridization wash. Cover slips were removed by hybridization buffer, sections washed in 37°C prewarmed solutions: 2 times 2xSSC + 10% formamide for 20 min, 2 times with 2xSSC for 15 min, followed by 2 times wash with 1xSSC for 15 min, then with 0.1xSSC for 15 min and, last, with 0.1xSSC for 5 min.

Thereafter, classical immunostaining was performed for insulin (Dako#A0546), the general lymphocyte marker CD45 (Dako#M0701) and VECTASHIELD® antifade mounting medium (Vector laboratories) including 4′,6-diamidin-2-phenylindol (DAPI). A 60× oil-immersion objective was used to acquire images images by a Nikon Ti MEA53200 (NIKON GmbH, Düsseldorf, Germany) microscope.

Quantification of cells and tissues

Morphometrical analyses of enteroviral mRNA, insulin, and CD45 were performed with a NikonTiMEA53200 (NIKON GmbH, Düsseldorf, Germany) microscope and NIS-Elements BR software. The number of virus-infected cells and number of islets and immune cells were counted manually throughout the whole sections. Viral RNA appeared as small dots within cells, which were separately counted for each cell by moving the z-focus of the microscope through each virus$^+$ cell. Cells were defined as “single infected” with 1–10 puncta or “fully infected” with ≥10 puncta. A representative picture of infected cells was taken in a way that most “puncta” could be seen. Mean β-cell area per pancreas was calculated as the ratio of insulin-positive to whole pancreatic tissue area. The exocrine area was calculated as whole pancreas area subtracted by the insulin-positive area. “Islet periphery” was

defined as signal localization within three cell layers next to insulin containing islets and "close proximity" as signal localization within three cell layers next to the respective islet or immune cells.

Statistical analyses

All biological replica referred to "n" for each individual human pancreas, which are means of two technical replicas from independent staining analyses and presented as means ± SEM. Mean differences were determined by the Mann–Whitney non-parametric two-tailed test, in which the whole T2D group was compared to the control group without diabetes. In a subgroup analysis (Supplementary Figure S3), either the Aab$^-$T2D group or the Aab$^+$-T2D group was compared to the control group without diabetes. A p-value <0.05 was considered statistically significant. Investigators were blinded to the cases.

Study approval

Ethical approval for the use of human pancreatic tissue had been granted by the Ethics Committee of the University of Bremen. The study complied with all relevant ethical regulations for work with human tissue for research purposes. Organ donors or next of kin provided written informed consent for organ donation for research (52).

Results

Viral infiltration showed large heterogeneity and found scattered throughout the pancreases with no significant difference between controls and T2D (Figures 1A–C, I; Supplementary Table S2). Only one single T2D donor presented an exceptionally high number of virus-expressing cells within the exocrine pancreas, reminiscent of viral infiltration in T1D (Figure 2) (51). The proportion of donors with cells harboring virus RNA within the pancreas were 73% (11 of 15) among controls, and 66% (19 of 29) among T2D donors. A direct comparison of the cohort with our previous analysis with organ donors with T1D (51) showed the difference in viral RNA in T2D and T1D (Supplementary Figure S1). Despite some heterogeneity in the numbers of infected cells, donors with T1D were all positive for viral RNA$^+$ cells in their pancreases (100%), and their quantification showed sevenfold more viral RNA$^+$ cells in T1D than in controls of this cohort.

Enteroviral RNA$^+$ cells were only very rarely seen in islets (one single cell in two control donors and in 4 T2D donors, respectively; Figure 1D). The observation that enteroviral$^+$ cells scattered throughout the whole pancreas was verified by normalizing virus$^+$ cells within (Figure 1D) or in the periphery of islets (Figure 1E) to β-cell area and virus$^+$ cells in the exocrine pancreas area to exocrine area (Figure 1F), all of which were similar in controls and T2D donors (Figures 1D–F).

Thereby, the islet periphery was defined as insulin$^-$ cells within three layers next to insulin$^+$ cells of the islets, in analogy to our previous study in T1D pancreases (51). In these three cell layers, we found many enteroviral$^+$ cells in T1D (51), but only few in T2D (Figure 1E).

Lymphocytes expressing viral RNA (virus$^+$/CD45$^+$ co-positive) were rare and had similar frequency in controls and T2D (Figure 1G). Interestingly, many CD45$^+$ lymphocytes were found in close proximity to a virus$^+$ cell in the exocrine area, suggesting an active immune process where virus$^+$ cells were recognized by immune cells (Figure 1I). However, as this study is limited to the use of fixed tissue, we were unable to verify such active process.

With the normalization of viruses to their cellular location and the quantification of CD45$^+$ lymphocytes and insulin$^+$ β cells in the pancreas we confirmed and verified the increase in lymphocytes and the reduction in β-cell area in T2D in this well characterized nPOD cohort (Figure 1H), in line with previous elegant studies (6, 7, 55–57). While there was heterogeneity among islets as well as among donor pancreases, β-cell area/exocrine area (previously also called β-cell volume) was reduced by 49% in the pancreases of donors with T2D, compared to controls (Figure 3), in line with results obtained from the Mayo clinic's cohort (7). The number of CD45$^+$immune cells localized in islets was twofold increase in T2D donors compared to controls without diabetes, analogously to previous observations in isolated islets (55) and in pancreas sections (57). With an average of 0.3 ± 0.03 CD45$^+$ cells per islet, islet lymphocytic infiltration in all donor pancreases of this study (including the donor with the exceptional high number of viruses; Figure 2) was much lower than the defined threshold of 15 CD45$^+$ cells/islet for T1D (58, 59), which confirms classification to T2D of cases analyzed in this study, despite the higher number of inner-islet-CD45$^+$ cells in T2D, compared to nondiabetic controls.

We found no correlation of enteroviruses with the mean number of islets, nor with β-cell area, nor with age of the donor (Supplementary Figure S2). Only the single donor with an exceptionally high virus counts of 925 virus-expressing cells throughout the pancreas had the lowest number of islets (mean of 80) in the observed pancreas sections (donor ID #6133; Figure 2; Supplementary Figures S1, S2), together with a low β-cell area (Supplementary Figure S2B). Most donors with a relatively high virus count of >20 enterovirus expressing cells throughout the pancreas section were in an age group >45 years (Supplementary Figure S2C; dashed box). Only the youngest donor of the whole cohort had a high virus count of 94.5, was already diagnosed with T2D at the age of 15, is severely obese (BMI of 37), and presented uncontrolled hyperglycaemia with an HbA1c of 10.7%. We compared these results with those of younger control donors from our previous analysis (51) (mean age of 21.5 years), and they also showed the very low number of <20 enterovirus$^+$ cells/section (Supplementary Figure S1C).

Of note, within the T2D group, we have also tested donors that had developed single T1D-associated antibodies against insulin (mIAA; n = 10, mean age = 47; Supplementary Table S1), most likely in response to subcutaneous insulin injection, as all T2D-IAA$^+$ donors had received insulin therapy before or during hospitalization. Two donors were positive for glutamic acid decarboxylase (GADA; n = 2; mean age = 53), which is an early T1D-autoantibody and marker of T1D progression (60), but all

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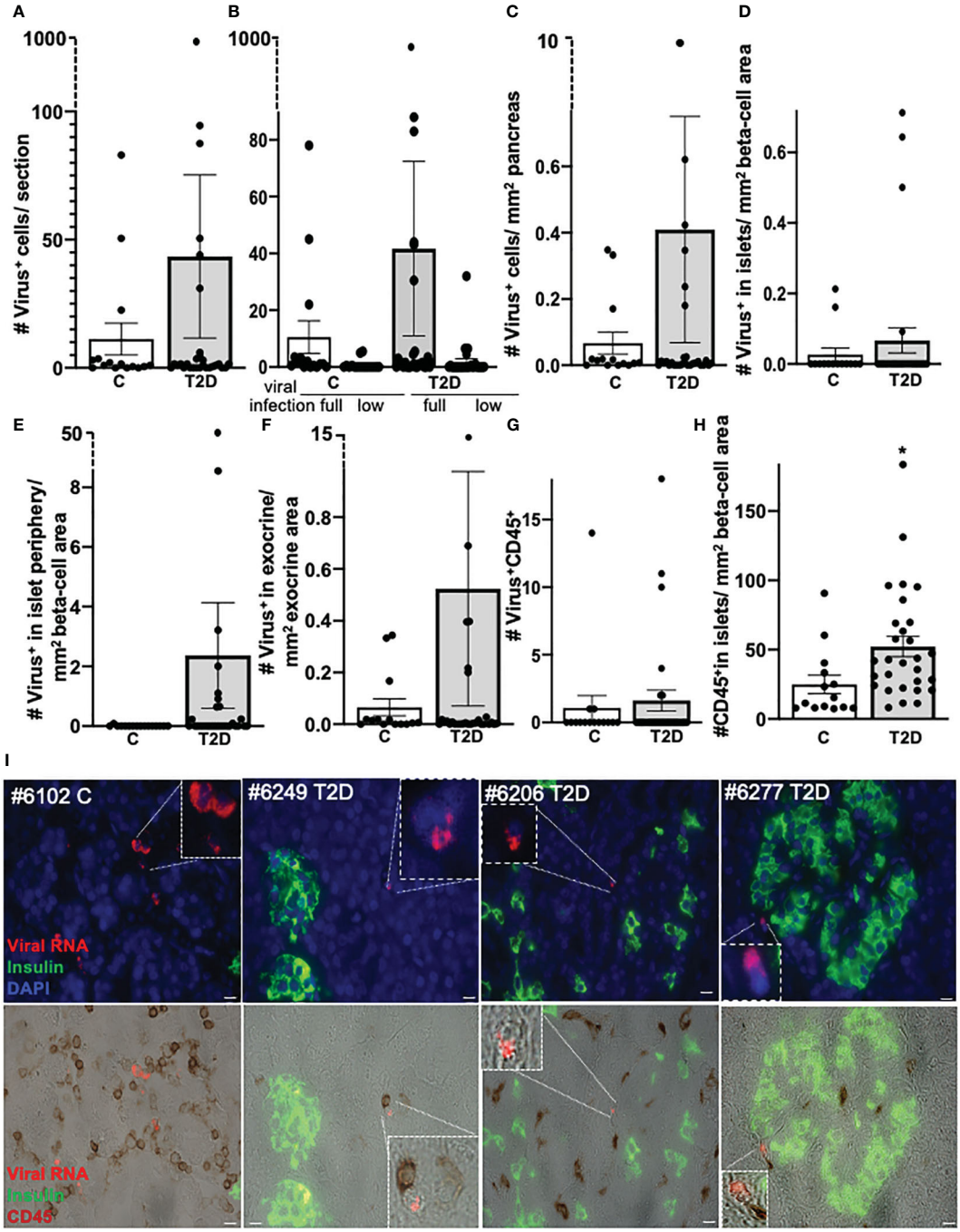

FIGURE 1
No correlation of pancreatic enteroviral RNA in organ donors with T2D and controls without diabetes. Detection and quantification of viral RNA in FFPE pancreases from control donors without diabetes (n = 15) and donors with T2D (n = 29). Data are presented as **(A)** mean number of all enteroviral RNA$^+$ cells throughout the whole pancreas section, **(B)** the mean number of low grade (low; one to nine single puncta/cell) or full-grade (full; ≥10 single puncta/cell) infected cells. In the latter, viral RNA highly accumulated in the cell and therefore could no longer be counted as single puncta. **(C)** All enteroviral mRNA$^+$ cells were normalized to the whole pancreas area of the respective section. **(D–F)** Enteroviral mRNA$^+$ cells within islets **(D)** and within the periphery of three cells next to insulin containing islets **(E)** were normalized to islet area (insulin$^+$ stained area in mm^2), and **(F)** viral mRNA$^+$ cells in the exocrine area were normalized to the mm^2 exocrine area of the respective section. **(G)** Quantification of enteroviral RNA$^+$/CD45 co-positive cells throughout the whole pancreas section and **(H)** of CD45$^+$cells within insulin containing islets normalized to mm^2 islet area. Each individual point of the scatter graphs represents the mean of two technical replica from each donor pancreas, boxes are means ± SEM from all donors. *P < 0.005 by Mann–Whitney non-parametric two-tailed test. **(I)** Representative microscopical pictures of enteroviral RNA in the pancreas. Quadruple immunostainings of enteroviral RNA (red), insulin (green), DAPI (blue, all upper), and CD45 (brown, lower) in FFPE pancreases from a control donor without diabetes (1) and three donors with T2D (2–4) and their localization within the exocrine pancreas (1–3) or within the periphery of three cells next to insulin containing islets (4) and their proximity to CD45$^+$ lymphocytes. Scale bars depict 10 µm. Magnifications show enteroviral RNA$^+$ cells.

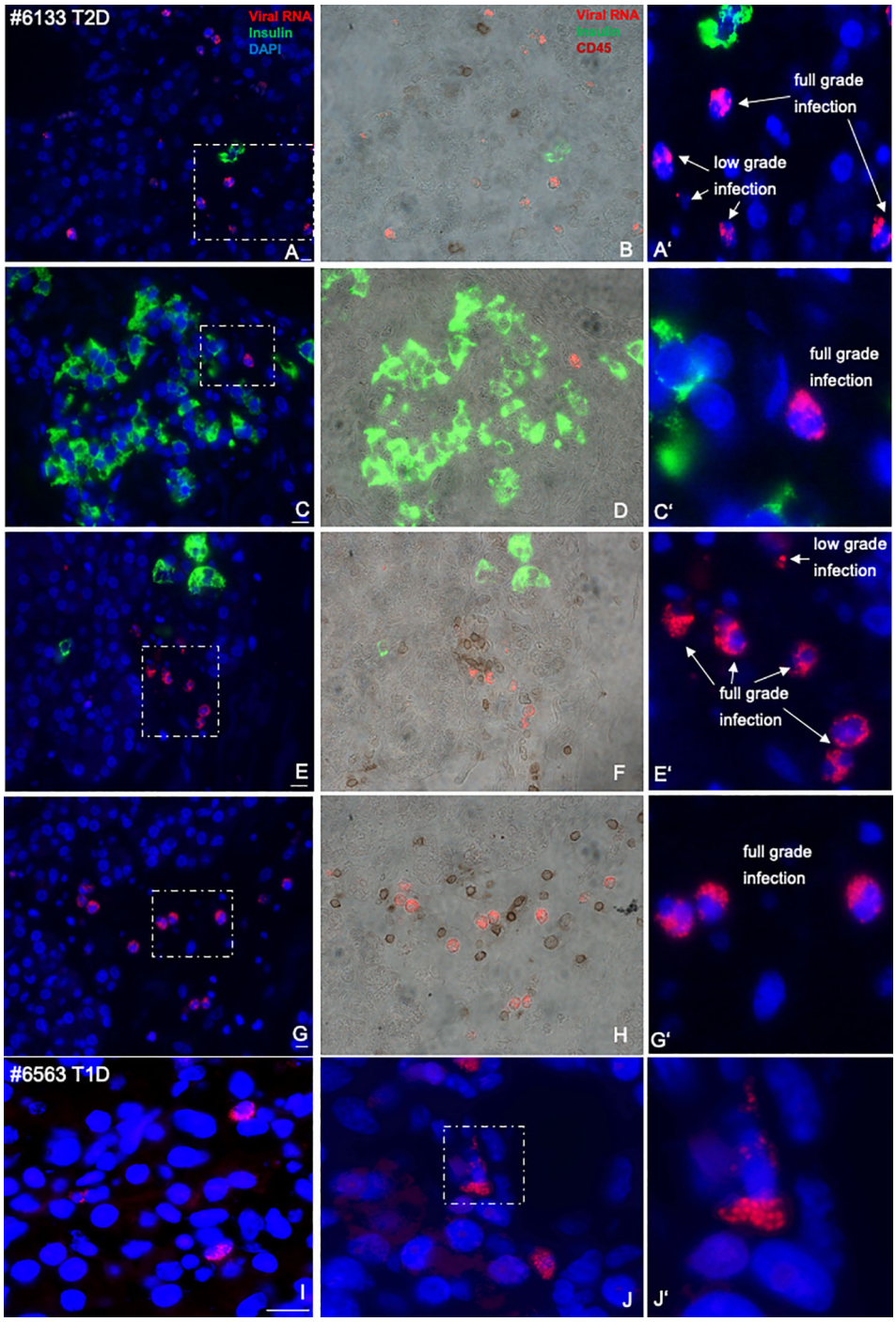

FIGURE 2
Representative microscopical pictures of pancreas sections from an nPOD donor with T2D with specifically high-enteroviral RNA and comparison with T1D. Quadruple immunostainings of enteroviral RNA (red), insulin (green), DAPI (blue, left **(A, C, E, G)** and CD45 (brown, middle **(B, D, F, H)** in an FFPE pancreas from a donor with T2D scattered throughout the pancreas in proximity to single β cells **(A, B)**, islets **(C, D)**, or scattered within the pancreas **(E–H)**, in proximity to $CD45^+$ lymphocytes or co-stained with CD45 **(B, F, H)**. Magnifications **(A', C', E', F')** show enteroviral RNA^+ cells with low- and full-grade infections, where viral RNA highly accumulated in the cell. Representative pictures of the exocrine region from a donor with T1D was included for comparison **(I, J, I')** in larger magnification. Representative pictures of infected cells were taken in a way that most "puncta" could been seen. Scale bars depict 10 µm.

these donors fulfilled the classification of T2D, based on pancreas morphology and c-peptide levels (59, 61). As we had previously seen a significant correlation of T1D-associated autoantibody positivity and enteroviral RNA in the pancreas in young donors (mean age of 20 years; maximum 26 years), we performed a sub-analysis of this category (Supplementary Figure S3) but could not find any significant difference in pancreatic viral RNA and their localization within the pancreas, when compared to controls

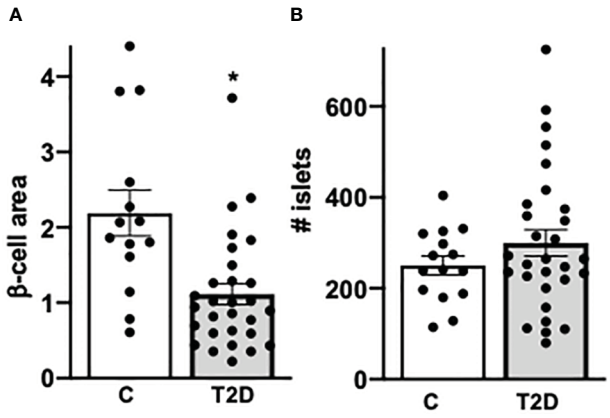

FIGURE 3
Confirmed decreased β-cell area in T2D. **(A)** For β-cell area analysis, the percentage of β cells were calculated by the ratio of mm^2 insulin area and mm^2 of the whole pancreas area from each section (previously also called β-cell volume). **(B)** The absolute number of islets was counted in each section. Each individual point of the scatter graphs represents the mean of two technical replica from each donor pancreas, boxes are means ± SEM from all donors. *P < 0.05 by Mann–Whitney non-parametric two-tailed test.

(Supplementary Figures S3A–D). In contrast to our previous study, single AAb^+ donors, which have developed T2D, did not show any differences in the number of enteroviral RNA in the pancreas, while young single and multiple AAb^+ donors (mean age of 20 years) without diabetes had a significantly increased enteroviral RNA in the pancreas, compared to nondiabetic controls. Here, only older donors (mean 48 years) with a single GADA- or IAA-AAb were analyzed, who had not progressed to classical T1D or late onset autoimmune diabetes in adults, their pancreases had many large islets and very few lymphocytes in islets (mean of 0.1 $CD45^+$cell/islet) and normal C-peptide levels (mean = 4.3 nmol/L). The increase in lymphocytes (Supplementary Figure S3E) and the reduction in β-cell area (Supplementary Figure S3F) were again confirmed in T2D, also when each of the two T2D groups with or without T1D-associated Aabs were independently compared to the control pancreases without diabetes.

Discussion

Altogether, in T2D, enteroviral RNA could be detected within the pancreas but found at similar levels to nondiabetic controls. This is in contrast to the established increased pancreatic enterovirus expression in T1D-associated autoantibody-positive individuals and in T1D, where viral infections contribute to abnormalities in both the endocrine and exocrine pancreas (51).

Enteroviral RNA was detected and quantified by the highly sensitive smFISH method, which had originally been developed to visualize each mRNA molecule as a computationally identifiable fluorescent spot by fluorescence microscopy (62). We had adapted smFISH for enteroviral RNA screening in the pancreas and called all positive spots "puncta" (51, 53). By using this method, Farack et al. identified transcriptional heterogeneity of β cells in the pancreas with some β cells containing much more insulin, which they called "extreme" β cells. In these cells, insulin mRNA could not be distinguished as puncta anymore but as large-signal accumulation (63). Similarly, viral RNA is seen as small fluorescent spots within cells, which were separately counted for each cell by moving the z-focus of the microscope through each $virus^+$ cell. Cells were defined as "single infected" with 1–10 puncta or "fully infected" with ≥10 puncta. In the latter, viral RNA had highly accumulated in the cell and could no longer exactly be counted as single puncta. Therefore, we had developed such threshold analysis of low and highly infected cells.

Comparison of our results with earlier studies which had analysed coxsackieviruses in the T2D diabetes pancreas reveals several important differences (1): the presence of virus in these studies was limited to viral capsid VP1 staining, which has a much lower sensitivity (53) and specificity (64) (2); VP1 had exclusively been analysed within islets, where viruses are very rare and the number and size of islets very heterogeneous; and (3) VP1 positivity in islets of each donor had only been based on qualitative results, and not on viral quantification, which excluded stringent statistical analysis.

While $viral^+$ cells within islets are a rare phenomenon, they were seen more frequently in proximity to the islets, that is, within three layers next to $insulin^+$ β cells in T1D and, even in this analysis in T2D, viral RNA^+ cells were 35-fold more frequent in the islet periphery than within islets and fivefold more than in the exocrine pancreas (then normalized to the respective area as presented in Figures 1D–F). Previous analyses suggest that such "peripheral cells" (either within or near islet cells) are more associated with a pathological phenotype than other islet cells. For example, using large-scale electron microscopy images ("nanotomy") of nPOD human pancreas tissue, de Boer et al. identified morphologically abnormal cells containing both endocrine and exocrine granules in organ donors with T1D. These cells could neither been characterized as endocrine nor as exocrine cells (65). Also, two important studies show β-cell heterogeneity markers with their expression reduced frequently at the islet periphery. Van der Meulen et al. observed unusual immature "virgin" urocortin (UCN)3-negative β cells at the islet periphery. While labeling specific plastic cells, which undergo transdifferentiation, UCN3 is one of the first β-cell genes, which is downregulated during β-cell failure and, thus, also marks dysfunctional and dedifferentiated β-cells (66, 67). Another heterogeneity gene, ST8Si1, is often seen lost at the islet periphery, although such $ST8Si1^-$ β cells are highly functional (68), and ST8Si1 expression is increased in T2D (68), possibly as part of the sialic acid-mediated immune response (69).

As viral infections promote β-cell dysfunction and dedifferentiation (70), several scenarios of the mechanisms of viral RNA presence in a subpopulation of cells in close islet neighborhood are possible; either infected cells have lost endocrine hormone expression and dedifferentiated, their specific phenotype makes them more vulnerable to viral infection or they hide from the immune system and thus remain a long time in the system. This may be a major path to diabetes pathology and remains to be investigated.

Independent of their diabetes state, it became apparent that most donors with a higher number of $virus^+$ cells in the pancreas

(>20) were from the age group 45+ years. With the age-dependent reduction in the immune response (71), it is possible that enteroviruses are not fully cleared in older individuals. Such hypothesis is in line with the increased vulnerability to infectious as well as metabolic diseases at an older age (71), and with a chronic low-grade inflammation, together referred to as "inflamm-aging."

An overall existence of a low grade persistent viral infection in the pancreas may contribute to the progression of β-cell destruction and T2D in vulnerable individuals over time. Enteroviral RNAs trigger long-term pathology in the heart such as cardiac dysfunction and cardiomyopathy (72), both part of the metabolic syndrome and T2D. While acute viral infection requires viral clearance through the immune system, viral RNAs remain persistently in few cells and may cause pathology in genetically predisposed individuals. If not primarily, it could trigger potentiation of inflammation. For example, MafA, a crucial transcription factor for β-cell function is remarkably decreased in T2D β cells and its reduction leads to critical changes in the β-cell anti-viral response and susceptibility to enterovirus infection (73). In response, levels of MafA and other β-cell functional markers are further reduced by β-cell dysfunction and inflammatory stress, which then leads to a vicious cycle with diabetes progression eventually. These mechanistical pathways came from *in-vitro* studies, in which virus effects could be studied directly.

Although we do not see differences in enteroviral RNA disposition in the pancreas from nondiabetic donors and those with T2D, the inflammatory process induced by infections during life may contribute to β-cell failure through various mechanisms and progression to T2D at an older age.

Author's note

The content and views expressed are the responsibility of the authors and do not necessarily reflect the official view of nPOD. Organ Procurement Organizations (OPO) partnering with nPOD to provide research resources are listed at https://npod.org/for-partners/npod-partners/."

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval for the use of human pancreatic tissue had been granted by the Ethics Committee of the University of Bremen. The study complied with all relevant ethical regulations for work with human tissue for research purposes. Organ donors are not identifiable and anonymous, such approved analyses using tissue for research is covered by the NIH Exemption 4 (Regulation PHS 398). Organ donors or next of kin provided written informed consent for organ donation for research (46).

Author contributions

HL performed experiments, analysed data and wrote the paper. SG designed and performed experiments and analysed data. AG, SS performed experiments, analysed data. AP provided intellectual support, pathological specimen and demographic data. KM designed experiments, analysed data, supervised the project and wrote the paper.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1236574/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Comparison of results from this study with pancreases from organ donors with T1D. Detection and quantification of viral RNA in FFPE pancreases from control donors without diabetes (n = 15), and donors with T2D (n = 29) from of this study and comparison with a previous study (51) of FFPE pancreases from donors with T1D (n = 15). The dashed box shows the T2D donor with >900 enteroviral RNA$^+$ cells. *P < 0.05 by Mann–Whitney non-parametric two-tailed test.

SUPPLEMENTARY FIGURE 2
No correlation of enteroviral$^+$ cells with β-cell area and age. In each graph, the number of all enteroviral RNA$^+$ cells throughout the whole pancreas section (see) were correlated with **(A)** the number of islets, **(B)** β-cell area, and **(C)** donor age. **(A–C)** All control (n = 15) and T2D (n = 29) organ donors of this study were included together with previously analyzed control nondiabetic organ donors from a younger cohort **(C)** n = 14; mean age of 21 years). The dashed boxes show donors with >20 enteroviral RNA$^+$ cells. Each individual point of the scatter graphs represents the mean of two technical replica from each donor pancreas, boxes are means ± SEM from all donors.

SUPPLEMENTARY FIGURE 3
Subgroup analyses of donors with T2D and single Aab$^+$ (from data in). The T2D cohort was divided into Aab$^-$ (n = 17) and single Aab$^+$ (n = 12) cases and subgroup analyses performed; each of the two subgroups was compared to the nondiabetic control pancreases (n = 15). **(A)** Detection and quantification of viral RNA in FFPE pancreases presented as **(A)** mean number of all enteroviral RNA$^+$ cells throughout the whole pancreas section. **(B)** All enteroviral mRNA$^+$ cells were normalized to the whole pancreas area of the respective section. **(C)** Enteroviral mRNA$^+$ cells within islets were normalized to islet area (insulin$^+$ stained area in mm^2). **(D)** Quantification of enteroviral RNA$^+$/CD45 co-positive cells throughout the whole pancreas section and **(E)** of CD45$^+$ cells within insulin containing islets normalized to mm^2 islet area. **(F)** For β-cell area analysis, the percentage of β cells were calculated by the ratio of mm^2 insulin area and mm^2 of the whole pancreas area from each section (previously also called β-cell volume). Each individual point of the scatter graphs represents the mean of two technical replica from each donor pancreas, boxes are means ± SEM from all donors. *P < 0.05 by Mann–Whitney non-parametric two-tailed test, in which either the Aab$^-$T2D group or the Aab$^+$-T2D group was compared to the control group without diabetes.

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Supplemental Figures

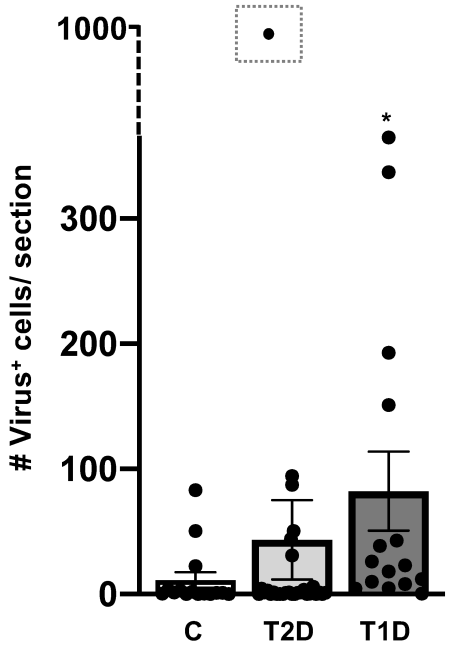

Figure S1

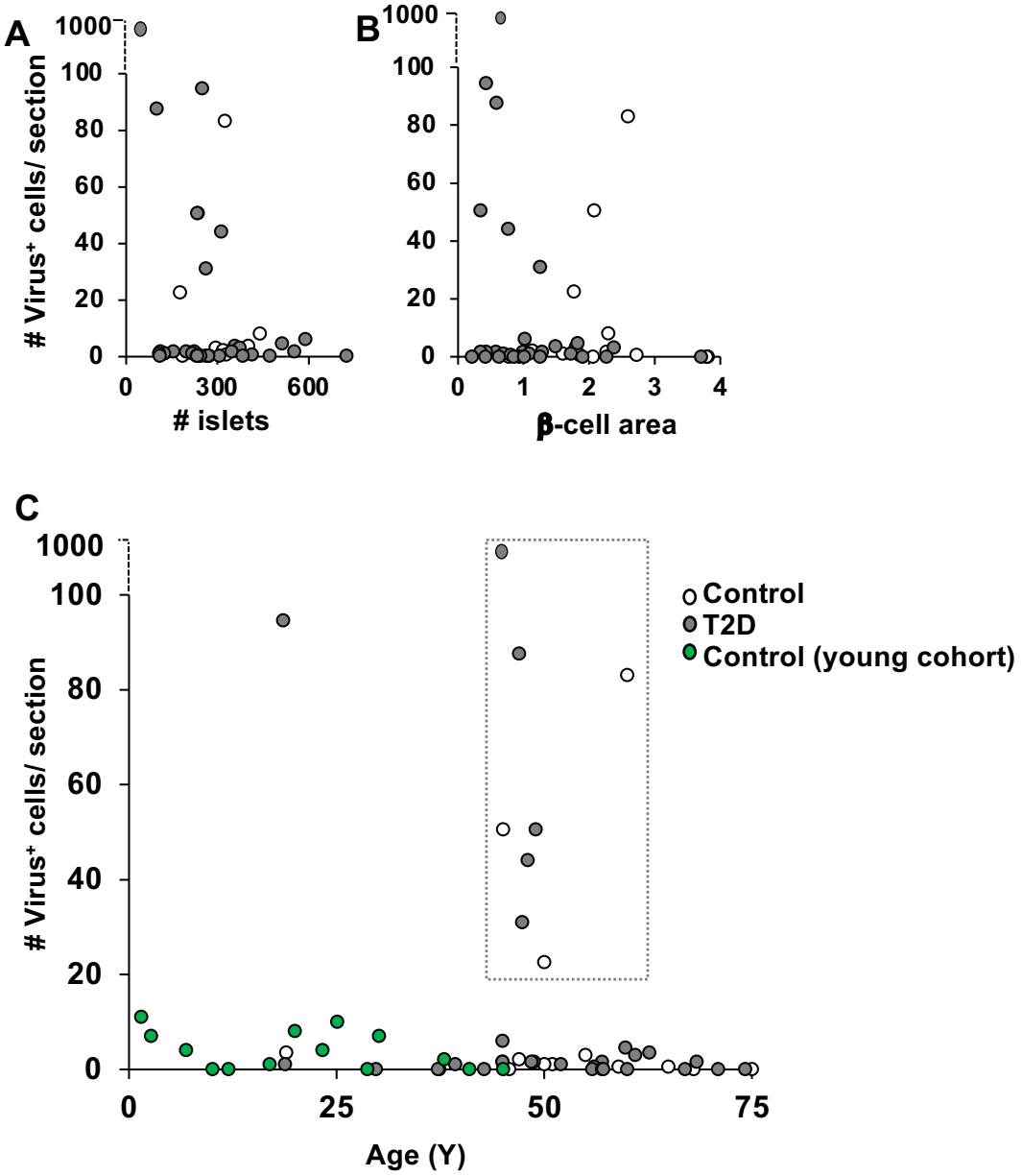
A
B
C
Virus+ cells/ section
islets
β-cell area
Age (Y)
Control
T2D
Control (young cohort)

Figure S2

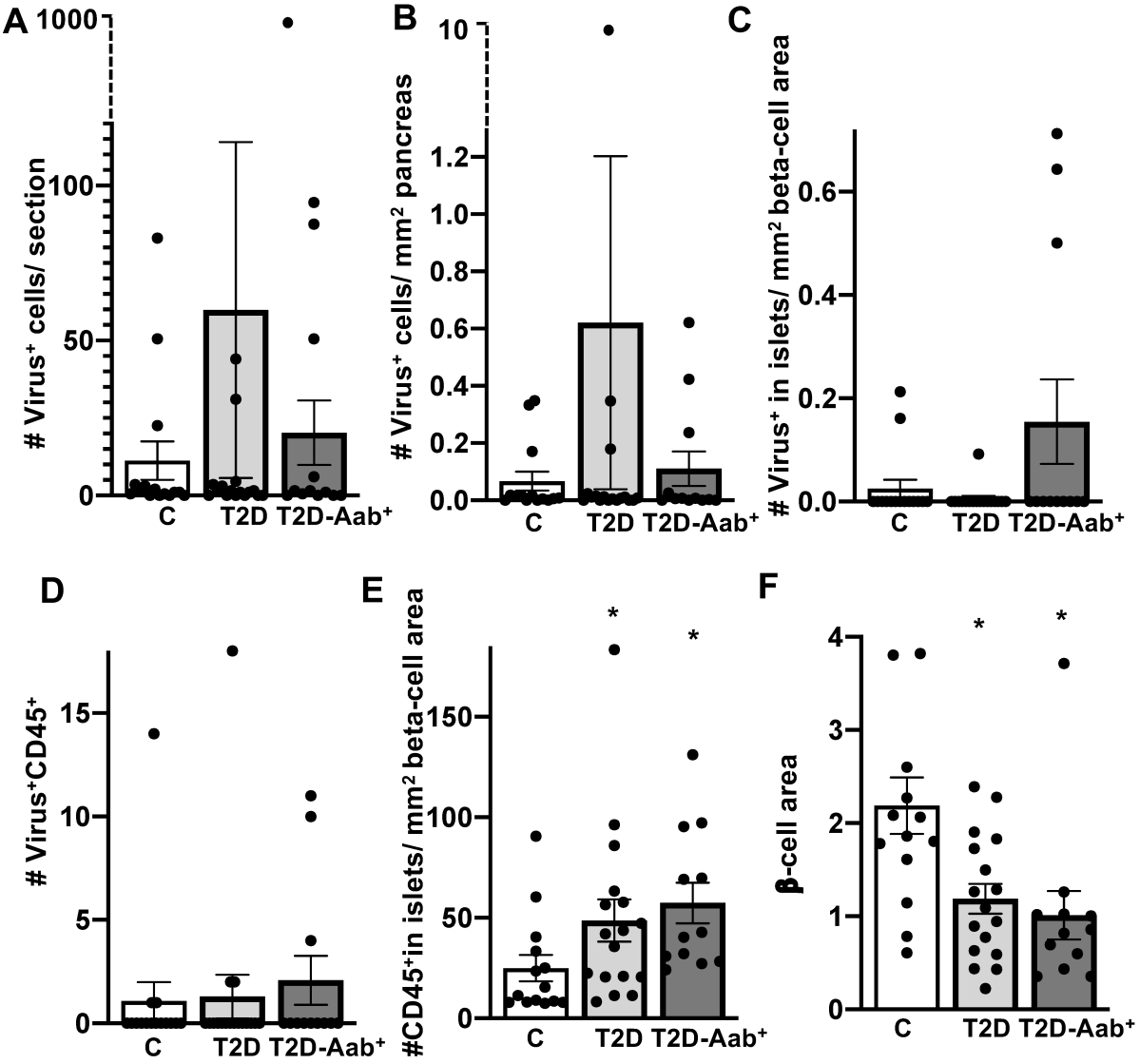
A
Virus⁺ cells/ section
1000
100
50
0
C
T2D
T2D-Aab⁺
B
Virus⁺ cells/ mm² pancreas
10
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C
T2D
T2D-Aab⁺
C
Virus⁺ in islets/ mm² beta-cell area
0.6
0.4
0.2
0.0
C
T2D
T2D-Aab⁺
D
Virus⁺CD45⁺
15
10
5
0
C
T2D
T2D-Aab⁺
E
#CD45⁺in islets/ mm² beta-cell area
150
100
50
0
*
*
C
T2D
T2D-Aab⁺
F
β-cell area
4
3
2
1
0
*
*
C
T2D
T2D-Aab⁺

Figure S3

Suppl.Table 1. nPOD inventory donor case IDs and demographics.

	ID	type	Aab	Gender	Ethnicity	DurDiabetes	HbA1c	C-pep.	CoD
1	6020	control	Aab-	male	Caucasian	0		2.82	Cerebrovascular/Stroke
2	6102	control	Aab-	female	Caucasian	0	6.1	0.55	Cerebrovascular/Stroke
3	6008	control	Aab-	female	Caucasian	0			head Trauma
4	6279	control	Aab-	male	Caucasian	0		8.01	Head Trauma
5	6288	control	Aab-	male	Caucasian	0		12.96	Head Trauma
6	6295	control	Aab-	female	African Am	0	5.3	10.91	Cerebrovascular/Stroke
7	6009	control	Aab-	male	Caucasian	0		11.32	Anoxia
8	6168	control	Aab-	male	Hispanic	0	6.2		Cerebrovascular/Stroke
9	8003	control	Aab-	male		0			Cerebrovascular/Stroke
10	6013	control	Aab-	male	Caucasian	0		2.80	Cerebrovascular/Stroke
11	6017	control	Aab-	female	Caucasian	0		9.89	Cerebrovascular/Stroke
12	6165	control	Aab-	female	Caucasian	0	5.6	4.45	Cerebrovascular/Stroke
13	6022	control	Aab-	male	Caucasian	0		4.99	Cerebrovascular/Stroke
14	6012	control	Aab-	female	Caucasian	0		2.97	Cerebrovascular/Stroke
15	6545	control	Aab-	female	Caucasian	0		1.78	Cerebrovascular/Stroke
MEAN				**7F/8M**		**0.0**	**5.8**	**6.1**	
1	6133	T2D	Aab-	female	Caucasian	20		0.84	Anoxia
2	6277	T2D	Aab-	male	African Am	10		0.47	Cerebrovascular/Stroke
3	6194	T2D	Aab-	male	Caucasian	13	7.3	0.16	Cerebrovascular/Stroke
4	6206	T2D	Aab-	male	Caucasian	10	8.5	11.15	Cerebrovascular/Stroke
5	6191	T2D	Aab-	female	Caucasian	10	6.0	6.14	Cerebrovascular/Stroke
6	6221	T2D	Aab-	female	Caucasian	4		3.05	Cerebrovascular/Stroke
7	6273	T2D	Aab-	female	African American	2		3.17	Anoxia
8	6272	T2D	Aab-	female	African American	10		7.55	Anoxia
9	6186	T2D	Aab-	male	Caucasian	5	6.3	2.98	Cerebrovascular/Stroke
10	6059	T2D	Aab-	female	Hispanic	0.25		10.68	Anoxia
11	6304	T2D	Aab-	female	Hispanic	25		2.34	Cerebrovascular/Stroke
12	6157	T2D	Aab-	female	African Am	1	6.9	2.74	Cerebrovascular/Stroke
13	6114	T2D	Aab-	male	Caucasian	2	7.8	0.58	Anoxia
14	6139	T2D	Aab-	female	Hispanic	1.5		0.6	Anoxia
15	6132	T2D	Aab-	female	Hispanic	0	9.1	0.80	Anoxia
16	6259	T2D	Aab-	male	Caucasian	10		1.31	Cerebrovascular/Stroke
17	6541	T2D	Aab-	male	Hispanic	1	11.1	3.41	Cerebrovascular/Stroke
18	6499	T2D	mIAA+*	male	Caucasian	3	10.7	0.96	Head Trauma
19	6280	T2D	mIAA+*	male	African Am	10		3.71	Cerebrovascular/Stroke
20	6329	T2D	mIAA+*	female	Hispanic	25	11.2	7.46	Anoxia
21	6249	T2D	mIAA+*	female	Asian	15		4.17	Cerebrovascular/Stroke
22	6109	T2D	mIAA+	female	Hispanic	0	8.0	0.025	Cerebrovascular/Stroke
23	6189	T2D	mIAA+*	female	Caucasian	26		1.85	Cerebrovascular/Stroke
24	6149	T2D	GADA+	female	African American	16		11.55	Cerebrovascular/Stroke
25	6283	T2D	mIAA+*	female	Caucasian	17	9.6	1.77	Cerebrovascular/Stroke
26	6300	T2D	GADA+	male	Hispanic	0	6.5	3.19	Anoxia
27	6297	T2D	mIAA+*	male	Caucasian	3		3.23	Anoxia
28	6142	T2D	mIAA+*	female	Hispanic	14		0.19	Infectious Disease - Bacterial Meningitis
29	6269	T2D	mIAA+*	male	African Am	5		13.91	Cerebrovascular/Stroke
MEAN				**17F/12M**		**8.9**	**8.4**	**3.8**	

Suppl.Table 2. Number of viral mRNA positive cells in the pancreas. Related to Fig.1. Mean number of all viral mRNA positive cells/slide in each donor throughout the pancreas and separated in cells with the appearance of ≥10 (full grade infection) or 1-9 single puncta per cell (low grade infection)

	ID	type	Virus (n)	full grade(≥10)	low grade (<10)
1	6020	control	83	78	5
2	6102	control	50.5	45	5.5
3	6008	control	22.5	22	0.5
4	6279	control	3.5	3.5	0
5	6288	control	3	3	0
6	6295	control	2	2	0
7	6009	control	1.5	1.5	0
8	6168	control	1	1	0
9	8003	control	1	1	0
10	6013	control	0.5	0.5	0
11	6017	control	0.5	0.5	0
12	6165	control	0	0	0
13	6022	control	0	0	0
14	6012	control	0	0	0
15	6545	control	0	0	0
MEAN			**11.3**	**10.5**	**0.7**
1	6133	T2D	925	893	32
2	6277	T2D	44	43	1
3	6194	T2D	31	30.5	0.5
4	6206	T2D	4.5	4.5	0
5	6191	T2D	3.5	3.5	0
6	6221	T2D	3	3	0
7	6273	T2D	1.5	1.5	0
8	6272	T2D	1.5	1.5	0
9	6186	T2D	1.5	1.5	0
10	6059	T2D	1	1	0
11	6304	T2D	1	1	0
12	6157-03	T2D	0	0	0
13	6114-08	T2D	0	0	0
14	6139	T2D	0	0	0
15	6132	T2D	0	0	0
16	6259	T2D	0	0	0
17	6541	T2D	0	0	0
18	6499	T2D	94.5	88	6.5
19	6280	T2D	87.5	83	4.5
20	6329	T2D	50.5	44	6.5
21	6249	T2D	6	5.5	0.5
22	6109	T2D	1.5	1.5	0
23	6189	T2D	1.5	1.5	0
24	6149	T2D	1	1	0
25	6283	T2D	0.5	0.5	0
26	6300	T2D	0	0	0
27	6297	T2D	0	0	0
28	6142	T2D	0	0	0
29	6269	T2D	0	0	0
MEAN			**43.5**	**41.7**	**1.8**

Manuscript II

The Hippo terminal effector YAP boosts enterovirus replication in type 1 diabetes

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My Contribution:

Designed, performed experiments, analysed data and assembled the figures for:
Figure 1 (H; partially).
Figure 2 (F, partially).
Figure 3 (C, D, E, F, J, K, L, M, N) and (H, I, P, W; partially)
Figure 4 (A, B, C, D, G, H, I, J, O, P) and (E, F, K, L, M, N; partially)
Sup.Figure 3 (E, F, G) and (D partially)
Sup.Figure 4 (B, C, D, E, H, I, K, L, N, O,) and (G, J; partially)
Contributed in writing the paper and design the graphical abstract.

The Hippo terminal effector YAP boosts enterovirus replication in type 1 diabetes

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Key words: Type 1 diabetes; Pancreas; Exocrine; Islet; Beta cells; Enteroviruses; Hippo pathway; YAP; MST1; Inflammation; Apoptosis.

Abstract

Type 1 diabetes (T1D) is highly associated with enteroviruses especially with coxsackieviruses B (CVBs) infections but cellular host factors and intrinsic signaling mechanisms that contribute to the initiation or acceleration of virus-induced islet autoimmunity and consequent β-cell destruction remain unclear. Here we show that the Hippo pathway terminal effector, Yes-associated Protein (YAP) is highly upregulated in both the exocrine and endocrine pancreas of T1D and at-risk autoantibody-positive (AAb^+) organ donors. YAP expression is correlated with CVB infections and many YAP-expressing cells show virus positivity or localization in close proximity to virus-infected cells in T1D and AAb^+ pancreases. Importantly, over-expression of YAP results in enhanced CVB replication and fosters CVB-induced islet inflammation and β-cell apoptosis, whereas its inhibition halts viral replication in both primary and immortalized pancreatic cells. Mechanistically, we found that YAP in complex with its transcription factor TEAD directly induce their own negative regulator, kinase MST1, and MST1 inhibition increased viral replication and diminished β-cell apoptosis. This constitutes a negative feedback loop in which the reciprocal antagonism between YAP and MST1 regulates viral replication and β-cell death during CVB infections. Our work uncovers an integral role for YAP as key host factor for enteroviral amplification in pancreatic cells, and has an important translational impact for the viral replication prevention in T1D.

Introduction

Type 1 diabetes (T1D) is a multi-factorial inflammatory disorder manifested by autoimmune destruction of insulin-producing pancreatic β-cells, initiated by islet autoimmunity, immune cell recruitment and infiltration (insulitis) and locally released pro-inflammatory cytokines and chemokines. This results in β-cell apoptosis and ultimate loss of insulin production and development of hyperglycemia [1]. Although genetic predisposition is universally accepted as a key determinant in the development of T1D, environmental factors play their part, either as potential triggers or accelerators. Especially enteroviruses of the Coxsackievirus B (CVB) family have been implicated in the initiation and progression of islet autoimmunity, directly leading to β-cell destruction [2, 3]. Enteroviruses are small non-enveloped positive single-stranded RNA viruses of the Picornaviridae family [4]. CVBs are highly effective in infecting isolated human islets; their RNA and capsid protein were found in both the endocrine and exocrine pancreas of biopsies from living adults with recent-onset T1D as well as in autopsy pancreases from individuals with T1D, leading to MHCI-hyperexpression, local inflammation and β-cell destruction [5-12]. These findings support the possibility that a persistent low-grade infection may contribute to T1D pathogenesis [13].

While most studies exclusively investigated enteroviral expression within islets, CVB infection has also been reported in the exocrine pancreas in donors with T1D [7, 8]. By using a single molecule-based fluorescent in situ hybridization (smFISH) method, we have recently shown that enteroviral RNA is substantially increased in pancreases from organ donors with T1D and with disease-associated autoantibodies (AAb^+) with the majority of virus-positive cells scattered in the exocrine pancreas [8] suggesting that the exocrine pancreas is persistently infected with enteroviruses. Infected regions outside of islets are wired by immune cells and may constitute a potential reservoir for the virus itself or for the ongoing inflammation to spread to islets.

Enteroviruses may contribute to the development of T1D by various mechanisms; through direct destruction of β-cells due to virus infection, through viral persistence and chronic stimulation and recruitment of immune cells to the islets which leads to local inflammation, β-cell injury and subsequent release of autoantigens, which then trigger autoreactive T-cell responses and ultimately primes β-cell death [2, 13] and through “molecular mimicry”, in which immune cell migration occurs through the similarity of viral and β-cell epitopes shared between viruses and β-cells. Similar hypotheses link autoimmune disease in general, but present data still do not provide clarity whether viruses directly initiate autoimmunity and target cell destruction or only accelerate this process [14].

In order to efficiently replicate, viruses hijack the cellular machinery and signaling pathways. While external and internal receptors for enterovirus entry and sensing are known [15, 16], the endogenous host factor(s), their regulation in response to virus infections, and the molecular

mechanisms which lead to excessive stimulation of the immune system remain elusive. Pathways which regulate host's cellular survival and proliferation may provide a transiently surviving cell to the virus to attack its replication machinery. One such classical evolutionarily conserved pathway that controls organ size and tissue homeostasis and cellular survival is the Hippo signaling, which is tightly linked to the pathophysiology of cancer and metabolic diseases [17, 18]. Yes-associated protein (YAP) is the transcriptional co-regulator and the major terminal effector of the Hippo pathway. The activity of YAP is mainly regulated through a phosphorylation-dependent inhibition mechanism by the Hippo central kinases, mammalian STE20-like protein kinase 1 and 2 (MST1/2) and large tumor suppressor 1 and 2 (LATS1/2). Upon MST1/2 activation by physiological or pathological signals, MST1/2 phosphorylate and activate the LATS1/2 kinases, which in turn directly phosphorylate YAP on multiple sites, leading to YAP inactivation through its cytoplasmic retention and/or its degradation by the proteasome machinery. In contrast, when Hippo signaling is inhibited, YAP can freely translocate into the nucleus where it interacts with several different transcription factors such as the TEA domain family members (TEAD) and stimulates the expression of genes responsible for cell turnover, differentiation and regeneration [17].

The Hippo pathway has major control over pancreas development as well as over islet β-cell survival, regeneration and function [19-21]. YAP is broadly expressed in pancreatic progenitor cells in the developing pancreas and is indispensable for pancreatic cell identity through directing cell fate decisions and organ morphogenesis [22, 23]. While YAP's presence maintains in the exocrine pancreas and is essential for its function and plasticity, its expression is extremely low or undetectable in terminally differentiated adult endocrine islets [24-26]. Importantly, we and others have previously shown that re-expression of active YAP induces human β-cell proliferation indicating that the absence of YAP in adult human β-cells corelates with their low-replication capacity and β-cell quiescence [26, 27].

Previous studies have linked YAP with innate immunity to balance host antiviral immune responses [28, 29]. Thus, we investigated YAP as potentially dysregulated factor and initiator of the immune disbalance in T1D, and determined the functional significance and molecular mechanisms of YAP in enteroviral replication starting in the exocrine pancreas and promoting islet inflammation and β-cell apoptosis.

Results

YAP is highly upregulated in the pancreas of T1D and AAb$^+$ organ donors

Based on the fact that YAP is expressed in the human exocrine pancreas and directly linked to innate immunity and host inflammatory responses, we first examined the endogenous expression of YAP in the exocrine pancreas. Immunohistochemistry (IHC) for YAP was performed and analyzed in paraffin-embedded pancreatic tissue from organ donors with T1D (n=15), AAb$^+$ (n=15) and age and BMI-matched non-diabetic controls (n=13) from a well-characterized cohort of organ donors from nPOD (Network for Pancreatic Organ Donors with Diabetes; Table S1) [30]. YAP protein expression represented as %YAP-positive area in the exocrine pancreas was significantly higher in T1D (mean 19.95%) than in AAb$^+$ (mean 14.09%) and nondiabetic individuals (mean 11.97%) (Figures 1A,B). Moreover, a modest but significant increase in YAP-positive area in exocrine regions was also observed in AAb$^+$ donors compared to nondiabetic controls (Figures 1A,B). Consistent with previous findings [24, 31], ductal and terminal-duct centro-acinar cells expressed the highest levels of YAP in the exocrine pancreas (Figure S1A). The majority of AAb$^+$ and T1D donors abundantly expressed YAP within centro-acinar and ductal cells, while much less ductal YAP expression was observed in non-diabetic controls (Figure S1A).

Endocrine islets, including β-cells, do not express YAP [26, 27]. To investigate whether inner-islet expression of YAP in T1D is induced, we quantified the number of YAP-positive cells within the islet area. YAP-positive cells were markedly higher in islets from T1D (mean 3.05%) donors than from AAb$^+$ (mean 1.78%) and from nondiabetic (mean 0.64%) donors. Also many inner-islet YAP-positive cells were seen in the pancreas of AAb$^+$ donors (Figures 1C,D and S1B). To examine the cellular origin of augmented YAP-positive cells in AAb$^+$ and T1D islets, tissue sections were stained for YAP and chromogranin, a late endocrine marker also expressed by islet cells which have lost hormone expression [32]. Consistent with the higher inner-islet YAP expression observed in T1D donors, also the percentage of YAP/chromogranin double-positive cells were significantly higher in islets from T1D donors (mean 0.62%) than in AAb$^+$ (mean 0.10%) or nondiabetic (mean 0.04%) donors (Figures 1E,F).

The higher YAP protein abundance stemmed from elevated *Yap* mRNA expression. Determined by the highly sensitive in situ hybridization (ISH) RNAscope method, *Yap1* mRNA levels were significantly increased in donors with AAb$^+$ (mean 1.8 puncta per cell) and T1D (mean 3.01) compared with nondiabetic controls (mean 1.37) (Figures 1G,H). In addition, the expression of *Yap1* was higher in pancreases from T1D compared to AAb$^+$ donors (Figures 1G,H). These data indicate a positive correlation between YAP mRNA and protein expression, and confirm the transcriptional hyperactivation of *Yap1* in AAb$^+$ donors before T1D diagnosis as well as in T1D. Importantly, exocrine YAP levels highly correlated with endocrine YAP expression in T1D (r=0.6964; p=0.005) donors, while there is a tendency also in AAb$^+$

(r=0.6242; p=0.060) (Figure 1I). These data indicate an association of changes not only in islets but also in the exocrine pancreas with T1D, and YAP upregulation as common modulator in both pancreas compartments. Testing a possible association between YAP expression and patients' clinical parameters revealed no correlation between YAP and age, BMI or Hb1AC in AAb^+ and T1D donors (Figures S1C-E).

YAP colocalizes and correlates with enteroviral RNA expression in the pancreas

Previous reports have identified YAP to balance host antiviral immune responses during viral infections [28, 29]. Therefore, we investigated the association between YAP expression and enteroviral infection in the pancreas of AAb^+ and T1D donors and YAP's cellular colocalization with two diabetogenic β-cell-tropic strains of CVB; CVB3 and CVB4 (CVB3/4) RNA. Double ISH-RNA analysis of *Yap* and CVB3/4 RNAs allowed us to systematically localize and quantify RNA throughout the whole pancreas sections. Due to the confirmed absent/very low number of virus-positive cells in the control group [8], such analysis was only possible in AAb^+ and T1D donors. Using single-cell analysis of CVB3/4 RNA and *Yap* mRNA stainings, we categorized infected cells into three groups: 1) cells with both YAP and viral RNA present in the same cell ("YAP^+/CVB^+"), 2) cells with viral RNA present in cells in close proximity of neighbor YAP-positive cells ("n-YAP^+/CVB^+") and 3) cells with no YAP but positive for viral RNA ("YAP^-/CVB^+"; Figure S2). *Yap* mRNA and enteroviral RNA mainly colocalized in the same cell, or Yap-positive cells were in close proximity to infected cells (Figures 2A-C). Quantification of double-positive pancreatic cells revealed the percentage of YAP/virus-co-positive cells were significantly higher in both patients with T1D as well as AAb^+ donors compared to YAP-negative/virus-positive cells (Figure 2C). Importantly, the mean number of YAP/virus-positive cells with both single (5-10 puncta per cell) and cluster (>10 puncta per cell) infections were markedly higher in T1D than AAb^+ donors (mean, 56 and 29 in T1D versus 31 and 7 in AAb^+ for single and cluster infections, respectively; Figure 2D) similar to what has been previously reported by us using the single-molecule *in situ* hybridization (smFISH) approach [8]. In addition to their cellular co-expression, YAP expression in the exocrine pancreas was positively correlated with the number of virus-expressing cells within the same region in AAb^+ donors (r=0.6193; p=0.08; Figure 2E). Likewise, YAP expression was moderately correlated with the number of virus-expressing cells in the pancreas of T1D donors (r=0.5149; p=0.06).

To confirm YAP-virus colocalization in the pancreas of AAb^+ and T1D donors at a single cell level, we complemented classical YAP-IHC staining with enteroviral RNA smFISH, which we have previously established to identify and localize enteroviral RNA in pancreata [33]. In line with CVB3/4-YAP RNA expression, YAP-protein/viral RNA double-positive cells were detected in AAb^+ and T1D donors (representative images shown in Figure 2F); most of the infected pancreatic cells expressed YAP. This suggests a pathological association between YAP and

enteroviruses and raises the question, whether the presence of YAP rather induces than balances enteroviral replication, and/or whether the infection *per se* may be a principal inducer of *Yap* transcription.

YAP enhances coxsackievirus replication and potentiates coxsackievirus- induced islet inflammation and β-cell apoptosis

To investigate a link between YAP and CVB infection and its functional significance on β-cells, they were infected with CVB3 and CVB4 (MOI of 5 and 10 for INS-1E β-cells and human islets respectively) [34, 35], together with the adenoviral mediated transduction of a constitutively active form of YAP (YAP-S127A). YAP overexpression was sufficient to enhance viral replication seen by the substantially increased CVB3 and CVB4 genomic RNA, relative to the control LacZ transduced in INS-1E cells (Figure 3A) and human islets (Figure 3B). The pro-viral effect of YAP was also confirmed by the increased level of the enterovirus-specific viral capsid protein VP1 upon YAP overexpression, compared to the control LacZ group in both INS-1E β-cells (Figures 3C,D) and human islets (Figures 3E,F). Immunofluorescence staining of VP1 and insulin verified the significant increase in the number of the VP1-positive β-cells by YAP overexpression in CVB-infected human islets, in comparison to control LacZ overexpression (Figures 3G,H). This supports the hypothesis that YAP hyper-activation potentiates viral replication. Further, microscopy analysis of infected cells revealed the abundant YAP/VP1/insulin triple-positive cells in primary human islets suggesting the cell-autonomous action of YAP (Figure S3A).

Besides β-cells, pancreatic exocrine cells and ductal cells in particular are highly susceptible to CVB infections [36]. As adult ductal cells naturally express YAP, we could investigate whether endogenous YAP has a similar pro-viral effect; we used verteporfin (VP), a chemical inhibitor of the YAP-TEAD complex [37], which blocked downstream actions of YAP. Immunofluorescent staining for VP1 and the ductal marker CK19 showed VP1-CK19 co-positive cells in both CVB3 and CVB4 infected human ductal cells (Figure 3I). The inhibition of YAP by VP led to inhibition of CVB3 and CVB4 replication as determined by the quantification of VP1/CK19 double-positive cells (Figures 3I,J). Similarly, YAP blockade by VP significantly abolished CVB4 RNA genome replication in the infected human ductal cell line PANC1 (Figure S3B). The efficiency of VP to inhibit YAP signaling was verified by mRNA analysis of YAP's target gene connective tissue growth factor (CTGF) [38], which was reduced by VP (Figure S3C). Consistently, VP also attenuated the number of VP1-positive cells, compared to control PANC1 cells only exposed to CVB4 (Figures S3D,E). These results suggested that YAP was indispensable for CVBs replication in both primary and immortalized ductal cells.

As YAP potentiates CVBs replication in both primary and immortalized β-cells, we further investigated whether this higher virus replication also increases apoptosis. CVBs highly induce

β-cell apoptosis [16]. YAP overexpression promoted a significant increase in CVB-mediated β-cell apoptosis as determined by caspase-3 cleavage, a universal marker of apoptosis, in INS-1E β-cells (Figures 3K,L) as well as in human islets (Figures 3M,N). TUNEL staining together with insulin confirmed the increased level of β-cell apoptosis in human islets upon YAP overexpression compared to LacZ-overexpressed controls (Figures 3O,P). Our data indicate an increase in viral replication together with virus-induced cell death, which would then lead to a higher rate of viral spread and a vicious cycle with viral progeny. Such hypothesis was confirmed by using polyinosinic-polycytidylic acid poly(I:C), a synthetic analog of double stranded RNA which mimics viral infection. YAP overexpression even declined poly(I:C)-induced β-cell apoptosis compared to the LacZ-transduced control group in INS-1E cells (Figures S3F,G) suggesting that the pro-apoptotic function of YAP fully depends on CVB replication.

As inflammatory/innate immunity responses mediate the pathophysiological mechanisms from enteroviral infection to T1D [39, 40] and YAP was shown to be linked to inflammatory reactions [41], we next assessed the impact of YAP on islet inflammation during CVB infections. In line with previous data [10, 39, 40], infection of human islets with CVB3 and CVB4 induced a strong type I interferon response represented by the mRNA upregulation of IFN-β (*IFNB1*) and the consequent production of IFN-stimulated genes (ISGs) including *CXCL10* and *OSA1* (Figures S3H-J) as well as by the Pattern recognition receptors (PRRs) and enteroviral sensors, such as RIG-I (*DDX58*), MDA-5 (*IFIH1*) and *TLR3* (Figures S3K-M). Indeed, YAP overexpression further enhanced not only *IFNB1* mRNA expression but also IFN-induced expression of *CXCL10* and *OSA1* (Figures 3Q-S) as well as of the PRRs *DDX58*, *IFIH1* and *TLR3*, compared to LacZ-transduced control cells (Figures 3T-V; the magnitude of the response varied between individual donors). In line with the gene expression data, overexpression of YAP potentiated the secretion of CXCL10 by infected human islets (Figure 3W). All these data indicated that YAP-overexpressing islets presented with higher level of antiviral response components.

A YAP-TEAD-MST1 feedback loop controls CVB replication and cell death

Dynamic and precise control of YAP activity by the upstream Hippo components is important to ensure proper cell stress response under physiological condition or upon invasion of pathogen. In the course of analyzing Hippo pathway, we have surprisingly noticed an increase in total MST1 protein level in the YAP-overexpressing INS-1E β-cells and human islets (Figures 4A-D), suggesting a novel Hippo feedback loop, in which YAP in its function as transcriptional co-regulator induces *STK4* (gene encoding MST1) transcription. Indeed, the amount of *STK4* mRNA was substantially increased in INS-1E cells overexpressing active YAP compared to control cells (Figure 4E). We then examined whether this feedback mechanism operates *in vivo* using β-cell specific YAP-overexpressing (β-YAP-OE) transgenic mice. In line

with data in cultured cells, a significant increase of *STK4* expression was evident in islets isolated from β-YAP-OE mice (Figure S4A) further supporting a role for YAP in *STK4* transcriptional regulation. Importantly, CVB4 infection itself triggered the induction of MST1 in both INS-1E cells and human islets (Figures S4B-E) suggesting that there may be a YAP-mediated feedback mechanism that occurs during CVB infection.

As YAP mostly acts through TEADs (TEAD1-4) transcription factors to regulate gene expression, we sought to mechanistically uncover the transcriptional regulatory activity of YAP/TEAD on MST1 (Figure S4F). The YAP-TEAD inhibitor VP reduced the transcriptional upregulation of *STK4* induced by YAP, compared to untreated INS-1E cells (Figure 4F). Consistently, VP fully reversed the induction of MST1 protein expression in YAP-overexpressing cells in both INS-1E cells (Figures 4G,H) and human islets (Figures 4I,J) in a dose-dependent manner. VP also triggered degradation of exogenous YAP as mechanism to block YAP downstream signaling (Figures 4G,I). The loss-of-function form of YAP with a S94A mutation abolishes its interaction with TEADs and therefore is transcriptionally inactive [38] and helps to further dissect the molecular basis of YAP/TEAD-mediated MST1 induction. Unlike the active form of YAP, overexpression of YAP-S94A mutant failed to induce MST1 at both mRNA and protein levels compared to the GFP-transfected INS-1E cells demonstrating that YAP stimulated MST1 in a TEAD-dependent manner (Figures S4G-I). Also, a genetically encoded fluorescently-tagged competitive inhibitor that blocks binding between YAP and TEAD ("TEAD inhibitor (TEADi") [42], attenuated *STK4* mRNA and MST1 protein levels in YAP-overexpressing cells (Figures S4J-L). Altogether, we conclude a YAP-TEAD mediated transcriptional induction of *STK4* and consequently elevated MST1 protein abundance, thus constituting a negativefeedback loop.

We then studied whether *STK4* is a direct transcriptional target of the YAP/TEAD complex. Two putative TEAD1-binding motifs were identified in the rat *STK4* promoter region by using a transcription factor-binding site prediction platform, the Eukaryotic Promoter Database (ED) [43] (Figure S4M). To experimentally confirm this, we used a luciferase reporter assay to examine whether the transcriptional rate of the *STK4* promoter could be stimulated by YAP. The *STK4* promoter region including a 1.5 kb sequence proximal to the transcription start site was cloned into an pEZX-PG04.1 reporter vector and transfected into Hela cells. We then generated a Hela cell line stably expressing conditional Gaussia Luciferase (GLuc) reporter located downstream of the *STK4* promoter and constitutively secreted Alkaline Phosphatase (SEAP) which was used as internal control for normalization. Dual reporter analysis showed that YAP overexpression significantly increased luciferase activity- as indicated by the ratio of secreted Gluc and SEAP-, compared to LacZ control, and this response was abolished by VP (Figure 4K). Chromatin immunoprecipitation (ChIP) coupled with qPCR (using two pairs of primers to amplify *STK4* promoter region) in INS-1E cells transduced with YAP or

corresponding LacZ control was conducted to check whether the YAP/TEAD transcriptional complex directly interacts with the promoter region of *STK4* gene. ChIP data using anti-YAP antibody and specific primers for the *STK4* promoter showed that YAP specifically binds to the *STK4* proximal promoter- as represented by fold enrichment in YAP occupancy- in INS-1E cells overexpressing YAP but not in the LacZ-overexpressing cells, which was again blocked by VP (Figures 4L,M). Positive control primers to amplify *ANKRD1*, a well-established direct target gene of the YAP/TEAD complex [44], and a negative control IgG verified ChIP specificity (Figures 4L-N). All these complementary methods indicate that the YAP/TEAD complex occupies the *STK4* promoter and exerts *STK4* expression induction in β-cells, confirming the postulated negative feedback loop.

To test the functional relevance of this YAP-MST1 loop during CVB infection, we performed MST1 knockdown experiments. SiRNA- mediated depletion of endogenous MST1 enhanced VP1 production, whereas at the same time attenuated apoptosis in CVB4-infected YAP-transduced cells, compared to control siScr transfected counterparts (Figures 4O,P). Consistently, immunofluorescence and qPCR analyses revealed that MST1 silencing in INS-1E cells resulted in significantly higher CVB4 replication as represented by increased VP1-positive infected cells in the siMST1-YAP-CVB4 group compared to the corresponding siScr-YAP-CVB4 control (Figures 4Q,R) as well as by increased intracellular CVB4 RNA genome (Figure 4S). To further confirm the anti-viral action of MST1, we used the dominant-negative form of MST1. Amino acid substitution mutation of the critical lysine within the ATP binding site (K59 for MST1) with alanine compromises MST1 kinase activity, thus MST1 is inhibited [45]. Infection of INS-1E cells transfected with MST1-K59 led to a marked enhancement of intracellular VP1 accumulation compared to the GFP-overexpressing cells, while inhibition of MST1 markedly attenuated the level of cleaved caspase-3 in YAP-overexpressing cells upon CVB4 infection (Figures S4N,O). Also, microscopy analysis of VP1-positive cells showed that MST1-K59 introduction stimulated an increase in CVB4 replication in INS-1E cells (Figures S4P,Q). Similar to the immunofluorescent staining, genetic MST1 antagonism largely induced the viral copies of CVB4 RNA compared to the GFP-transfected control group (Figure S4R) further indicating that MST1 blocks CVB4 replication. Given that MST1 is an upstream inhibitor of YAP in the classical Hippo cascade, and activated YAP induced the expression of MST1, YAP-mediated MST1 upregulation might at the end serve as a negative feedback loop to limit excessive YAP hyper-activation and subsequent CVB replication and amplification suggesting that the YAP-MST1 feedback mechanism plays an important role in regulating the viral replication machinery.

Discussion

There is a strong correlation of enterovirus infections with the onset of T1D, however, the molecular basis of the complex enteroviral-host interaction in the pancreas and how its dysregulation may lead to autoimmunity and T1D remain elusive. In this study, we show that YAP, a principal transcriptional effector of the Hippo pathway, is highly upregulated in both the exocrine and endocrine pancreas of AAb^+ and T1D organ donors. YAP expression is associated with enteroviral infections; the majority of CVB-infected pancreatic cells is either colocalized with YAP or located in close proximity to YAP-positive cells in AAb^+ and T1D pancreases. Cell-culture models of β-cells, human islets as well as human exocrine pancreatic cells show that YAP hyperactivation directly fosters CVB replication, potentiates β-cell apoptosis and enhances the expression of genes involved in innate immunity and antiviral defense. Conversely, pharmacological targeting of YAP blocks CVBs replication in YAP-expressing primary and immortalized pancreatic exocrine cells. Our detailed mechanistic work is the first to report MST1 as a direct YAP/TEAD target forming a cell-intrinsic feedback loop. This YAP-MST1 bidirectional interaction may acts as “molecular brake” to restrict excessive YAP-driven viral replication and amplification, to promote discarding infected host cells and to finally put the viral replication machinery on hold (Figure S5). Thus, we identified YAP is a pro-enteroviral factor, while MST1 has an antiviral function. This seems neither specific to pancreatic exocrine and endocrine cells nor to CVBs. YAP also promotes viral replication and production during SARS-CoV-2 or influenza infections [46, 47], while MST1 inhibits SARS-CoV-2 replication [46]. Accordingly, MST1 genetic deficiency enhances the susceptibility to pathogen infections as well as presents with autoimmune symptoms (e.g., hypergammaglobulinemia and autoantibody production) [48-51].

An imbalance between immune activation and immune protection is a key pathological element of autoimmune diseases such as T1D. Previous investigations highlighted the important regulatory function of YAP in inflammatory signaling. While highly complex and context- and cell type-dependent, its dysregulation is connected to inflammatory-related disorders such as atherosclerosis, non-alcoholic steatohepatitis (NASH), inflammatory bowel disease, pancreatitis and pancreatic cancer [41]. For example, YAP balances inflammation and supports tissue regeneration and repair, as *Yap* mRNA therapy improves cardiac function through anti-inflammatory mechanism in ischemia-reperfusion injury [52], or it blocks antiviral signaling to balance the host response which is vital for cellular survival during infection [28, 29]. On the contrary, YAP can also be pro-inflammatory i.e., YAP drives hepatic inflammation in NASH [53], and as we show, YAP is positive regulator of islet inflammation during CVB infection with an exaggerated interferon response that could initiate autoimmunity and loss of pancreatic cells as well as β-cells in T1D. In that line, YAP genetic loss in pancreatic neoplastic epithelial cells results in a decrease in the number of $CD45^+$ immune cells in the pancreas,

together with the progression of pancreatic ductal adenocarcinoma (PDAC) [31]. Likewise, YAP antagonism blocks the secretion of pro-inflammatory cytokines by neoplastic cells [31]. However, whether YAP controls inflammatory cell infiltration and inflammatory cytokine activation in preclinical mouse or human T1D requires further investigations such as using YAP-loss-of-function intervention approaches.

The innate antiviral immunity i.e., the IFN response is a key event in the course of autoimmunity and β-cell destruction. Type I IFN in islets triggers human leukocyte antigen I (HLA-I) [54], and HLA-I hyperexpression a hallmark of T1D [55]. The transcriptional signature of IFN responses precedes islet autoimmunity [56], and several polymorphisms within the interferon signature are genetic risk factors for T1D [57, 58]. In fact, incubation of islets with type I and III IFNs or boosting IFN response limits viral replication and associated cell injury in pancreatic islets [59, 60]. Nevertheless, if the activation of the IFN response is excessively prolonged or intense, it can also trigger autoimmune reactions in the islets and cause damage to β-cells. Interestingly, YAP has been implicated in innate immunity and was previously shown to negatively regulate the type I IFN response through blockade of antiviral signaling proteins TBK1 and/or IRF3 [28, 29]. Thus, our finding here, that YAP upregulated the interferon response during CVB infection in the pancreas is somewhat paradoxical, given YAP's inhibitory action on the antiviral response. One explanation for this paradox could be that the higher innate immune/antiviral response observed in YAP-overexpressing cells is primarily derived from an insufficient eradication of the virus (possibly through existing genetic polymorphisms in the interferon signature). Another possibility is that MST1, YAP's target gene identified in our study, enhances the antiviral response by (1) classical inactivation of YAP which would relieve the TBK1/IRF3 suppression, (2) direct activation of IRF3 as reported before in a different context [61], or (3) degradation of IRAK1, a negative regulator of type 1 IFN signaling [62]. In any case, such boosted antiviral response is unable to protect YAP-overexpressing cells against cell death caused by massive viral replication indicating that the classical intrinsic regulatory function of YAP/MST1 in antiviral signaling is overridden by the YAP-driven CVB amplification. In support of this argument, unlike in actual infections, YAP did not potentiate β-cell apoptosis in poly(I:C)-treated cells which confirms that cell death and lysis during CVB infection is a consequence of high viral replication. Similarly, UV-inactivated CVB virus is not able to kill β-cells [16], and the induction of proinflammatory cytokines and chemokines depends on viral replication [39].

Aberrant upregulation of YAP- marked by robust cytoplasmic and nuclear localization of YAP in ductal and centro-acinar cells- is not limited to the pancreas in T1D; other pancreatic disorders, including PDAC and pancreatitis present elevated expression of YAP [63-65]. YAP and its well-known target gene CTGF are robustly increased in pancreatitis [25, 65-67], an inflammatory disease of the exocrine pancreas manifested by extensive loss of the normal

exocrine parenchyma, fibrosis and inflammation, and both exocrine and endocrine functional failure. Commonly upregulated YAP in T1D as well as in PDAC and pancreatitis suggests that the Hippo/YAP pathway may play a general and central role in the pathogenesis of pancreatic disorders. Supported by using genetically engineered mouse models, pancreas-specific deletion of MST1/2 or LATS1/2, which is functionally equivalent to YAP activation, recapitulate T1D, PDAC or pancreatitis in terms of robust immune cell infiltration, widespread inflammation, fibrosis, reduced pancreas mass, exocrine dysfunction and disrupted islet architecture [24, 25, 66]. Importantly, genetic loss of YAP or CTGF neutralization is sufficient to rescue the phenotype [25, 66] indicating that YAP is a key driver of such pancreatic structural and functional abnormalities. Notably, various environmental, and metabolic factors, e.g., viral infections, inflammation, obesity, or diabetes have the potential to induce PDAC or pancreatitis [68, 69]. Also, a significant number of patients diagnosed with PDAC or pancreatitis have impaired glucose tolerance or diabetes [70, 71]. Although these pancreatic disorders differ mechanistically and phenotypically in many ways, YAP may function as a major hub of transcriptional convergence in the crosstalk between pancreatic cells and immune cells in response to microenvironmental cues such as infections or cellular transformation upon injury. YAP signaling could therefore be an important therapeutic target for pancreatic comorbidity disorders.

While the classical perspective regards T1D as a β-cell specific disease, recent findings indicate that T1D is a disorder that involves the entire pancreas in which the loss of functional β-cell mass is most evident [72, 73], together with the decreased pancreas mass [72, 73], immune cell infiltration and inflammation of the exocrine pancreas [74, 75], and exocrine dysfunction/insufficiency [76, 77]. An abnormal exocrine-endocrine cell interplay has previously been linked to the development of MODY8, a monogenic form of diabetes inherited in a dominant manner, in which a mutant gene expressed selectively in acinar cells induces impaired β-cell function and loss [78]. In a recent study, we have systematically shown the predominant presence of enteroviral RNA in the exocrine pancreas in patients with T1D [8]. This suggests that enteroviruses do not primarily target islet cells but the whole pancreas providing a pathological connection between T1D-related changes in the exocrine pancreas and the development of disease. Enteroviral infections in the exocrine pancreas can induce fulminant T1D marked by extensive inflammation with inflamed (CXCL10-positive) and/or infected (VP1-positive) ductal and acinar cells surrounded by immune cells such as T-cells indicating the existence of non-neglectable immune responses to enteroviral infection and subsequent cell injury in the exocrine pancreas [79]. In line with this, previous studies reported that, in addition to islets [80], CXCL10 expression is induced in the exocrine tissue in T1D [81] and gene expression analyses show the robust antiviral signature mainly in the exocrine pancreas in T1D [82].

A dysregulated/abnormal crosstalk between the exocrine and endocrine pancreas may have a more important role in the development of T1D than previously believed. Persistently infected exocrine cells in the pancreas where viral replication is promoted by YAP could be a potential trigger for a constant immune cell attack and the subsequent development of T1D in two ways: firstly, the persistently infected exocrine cells may act as "cellular reservoirs" that enhance viral replication in the pancreas, leading to higher viral loads and more efficient spread of the virus to the islet cells; and secondly, local inflammation triggered by the infected exocrine cells may directly harm β-cells and attract immune cells to infiltrate the islets, ultimately leading to the destruction of β-cells. Such complex exocrine-islet interactions require further mechanistic investigations- with major emphasis on immune cell responses and paracrine factors, in analogy with other pancreatic diseases. They will be key for targeted interventions for T1D.

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Author contributions

Conceptualization, Experimental design A.A.; Methodology S.G., K.M. and A.A.; Investigation S.G., H.L., D.G., M.K.M., A.M.G., D.B., B.L. and A.A.; Formal Analysis S.G., H.L., K.M. and A.A.; Writing - Original Draft A.A.; Writing - Review & Editing S.G., H.L., Z.A., A.P. and K.M.; Resources A.P.; Funding Acquisition and Supervision K.M. and A.A.

Declaration of interests

The authors declare no competing interests.

Methods

Cell culture, treatment and islet isolation

Human islets were isolated from pancreases of nondiabetic organ donors (both male and female) at University of Lille and ProdoLabs and cultured on Biocoat Collagen I coated dishes (#356400, Corning, ME, USA). The clonal rat β-cell line INS-1E was kindly provided by Claes Wollheim (Geneva & Lund University). The immortalized cell line Hela was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The human pancreatic exocrine ductal cell line PANC-1 was generously provided by Manfred Radmacher (Institute of Biophysics, University of Bremen). PANC-1 cells were cultured in complete DMEM (Invitrogen, CA, USA) medium at 25 mM glucose. Human islets were cultured in complete CMRL-1066 (Invitrogen) medium at 5.5 mM glucose. Hela and INS-1E cells were cultured in complete RPMI-1640 (Sigma Aldrich, Missouri, MO, USA) medium at 11.1 mM glucose. All media included with L-glutamate, 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). INS-1E medium was supplemented with 10 mM HEPES, 1 mM sodium pyruvate and 50-µM β-mercaptoethanol. In some experiments, human islets, INS-1E cells and PANC-1 cells were additionally cultured with 1-5 µM YAP/TEAD inhibitor verteporfin (#SML0534, Sigma Aldrich, USA) for 6h-24h. INS-1E cells were cultured with 2 µg poly(I:C) for 24h (#P9582; Sigma Aldrich). Hela cells were cultured with 2 µg puromycin-dihydrochlorid (P9620, Sigma, USA) for positive clonal selection.

All human islet experiments were performed in the islet biology laboratory, University of Bremen. Ethical approval for the use of human islets and formalin-fixed paraffin-embedded (FFPE) pancreatic tissue sections obtained from well-characterized organ donors from the network for Pancreatic Organ Donors with diabetes (nPOD) [30] had been granted by the Ethics Committee of the University of Bremen. The study complied with all relevant ethical regulations for work with human cells for research purposes. Organ donors are not identifiable and anonymous, such approved experiments using human islet cells for research is covered by the NIH Exemption 4 (Regulation PHS 398). Human islets were distributed by the two JDRF and NIH supported approved coordination programs in Europe (Islet for Basic Research program;

European Consortium for Islet Transplantation ECIT) and in the US (Integrated Islet Distribution Program IIDP).

YAP-transgenic mice and islet isolation

β-cell-specific YAP overexpressing (YAP-OE) mice were generated by crossing inducible aYAP overexpressing mice (TetO-YAPSer127A, provided to our lab in collaboration with Fernando Camargo, Boston Children's Hospital, Boston, MA) [83] with mice carrying the tetracycline transactivator (tTA) under the control of the insulin promoter (RIP-rtTA mice, kindly provided by Al Powers, Vanderbilt University Medical Center, Nashville, TN, USA) [84]. In the Rip-Ins2-TetO-hYAP1-S127A mice, rtTA gene becomes activated specifically in the islet β-cells due to the Ins2 promoter. Upon doxycycline (a tetracycline analog) treatment, the rtTA protein in these cells can bind to the tet-response element (TRE) and subsequently causing the transcription of the constitutively active form of YAP gene which is under a CMV promoter element. This system enables a fine-tuned spatio-temporal control over the expression of the aYAP gene in the pancreatic β-cells. All the experiments were done on 8-10 weeks old mice and genotype of the mice is in heterozygous condition. Pancreatic islets were isolated after 2 weeks doxycycline induction through drinking water in the mice. Islets from β-cell specific YAP-OE and respective control mice were isolated by pancreas perfusion with a Liberase TM (#05401119001, Roche, Mannheim, Germany) solution [80] according to the manufacturer's instructions and digested at 37°C, followed by washing and handpicking.

All mice used in this experiment were housed in a temperature-controlled room with a 12-h light-dark cycle and were allowed free access to food and water in agreement with NIH animal care guidelines, §8 German animal protection law, German animal welfare legislation and with the guidelines of the Society of Laboratory Animals (GV-SOLAS) and the Federation of Laboratory Animal Science Associations (FELASA). All protocols were approved by the Bremen Senate (Senator for Science, Health and consumer protection) and we have complied with all relevant ethical regulations for animal testing and research.

Viruses and virus purification and titration

Enteroviruses CVB3 (Nancy) and CVB4 (JVB) were kindly provided by Andreas Dotzauer (University of Bremen, Germany). Fetal Rhesus Kidney-4 (FRhk-4) cell line was used for the preparation and isolation of virus stocks. FRhk-4 cells were infected with CVB3 or CVB4 viruses for 2h and were cultured for 2-3 days until visualization of the cytopathic effect. The supernatant from these cells was harvested after 3 rounds of freezing and thawing followed by centrifugation for 10min at 720xg to precipitate cell debris. Virus purification was carried out by the sucrose gradient method using an ultracentrifuge. First supernatant was centrifuged at 4500 x g for 10min. Further, it was centrifuged for 12h at 120000 x g in 40% sucrose gradient buffer (40% sucrose, 10 mM Tris pH 7.5 100 mM NaCl and 1 mM EDTA). The invisible pellet was resuspended in 1x PBS. Aliquoted viral stocks were stored at −80 ℃. The TCID50 (tissue

culture infectious dose 50%) was determined using serial dilutions. Briefly, FRhK-4 cells were seeded in duplicates in 96-well plates. They were infected for 2h in serum-free media with serial dilutions of viral stocks. The cytopathic effect was determined under a light microscope and the TCID50 was calculated accordingly to Spearman-Kärber.

Virus infection of human islets or cell line

INS-1E or PANC-1 cells were infected with CVB4 virus at MOI (multiplicity of infection) of 5 in 35 mm dishes. Virus stocks were diluted in FCS free medium and cells were inoculated with 750 µl at 37°C and 5% CO2. Control cells were incubated only with 750 µl of FCS-free medium. After 2h infection, cells were washed three times with 1xPBS and media was replaced by 10% FCS supplemented media for 24h. Infection of Human islets was performed with CVB3 or CVB4 viruses at MOI 10 under the same condition. For human islets 48h post-infection endpoint was chosen and then cells were harvested for staining as well as protein or RNA analysis. The culture supernatants were collected for measuring secreted CXCL10.

Adenovirus transduction

The adenoviruses control Ad-CMV-b-Gal/LacZ (#1080) and Ad-CMV-h-YAP1-S127 (custom production) were purchased from VECTOR BIOLABS, PA, USA. Isolated human islets or INS-1E cells were infected with Ad-LacZ or Ad-YAP at a multiplicity of infection (MOI) of 100 (for human islets) or 10 (for INS-1E) for 4h in CMRL-1066 or RPMI-1640 medium without FBS respectively. After 4h incubation, adenoviruses were washed off with PBS and replaced by fresh complete medium which contains 10% FBS. Human islets or INS-1E cells were collected for staining, as well as RNA and protein isolation after 48-72h transduction.

Plasmids and siRNAs

To knock down MST1, SMARTpool technology was used (Dharmacon, CO, USA). A mix of ON-TARGETplus siRNAs directed against the following sequences: rat MST1 (#L-093629-02) sequences CUCCGAAACAAGACGUUAA; CGGCAGAAAUACCGCUCCA; CGAGAUAUCAAGGCGGGAA; GGAUGGAGACUACGAGUUU. An ON-TARGETplus nontargeting siRNA pool (Scramble; siScr) served as controls.

Following plasmids have been used: Kinase-dead (MST1-K59; dnMST1) was kindly provided by Dr. Junichi Sadoshima and Dr. Yasuhiro Maejima (UMDNJ, New Jersey Medical School). pCMV-Flag-YAP-S94A was a gift from Kunliang Guan (Addgene plasmid # 33102; http://n2t.net/addgene:33102 ; RRID:Addgene_33102) [38]. pCEFL EGFP-TEADi was a gift from Ramiro Iglesias-Bartolome (Addgene plasmid # 140144 ; http://n2t.net/addgene:140144 ; RRID:Addgene_140144) [42]. pCMV-flag S127A YAP was a gift from Kunliang Guan (Addgene plasmid # 27370 ; http://n2t.net/addgene:27370 ; RRID:Addgene_27370) [85]. GFP plasmid was used as a control.

Transfection

GFP, EGFP-TEADi, MST1-K59, YAP-S94A, and pCMV-flag S127A YAP plasmids were used to overexpress these proteins in INS-1E cells. 100 nM MST1 or scr siRNAs were used for the transfection in INS-1E cells. To achieve silencing and overexpression, jetPRIME® transfection reagent (#114-75; Polyplus transfection, France) was used to deliver desired siRNA or DNA into INS-1E cells according to manufacturer's instructions. In brief, jetPRIME buffer was mixed with siRNA/DNA and vortexed for 10s, then jetPRIME® transfection reagent was added and vortexed for 1s. The mixture was stand at room temperature (RT) for 10 minutes after quick spin. The jetPRIME-siRNA or DNA complexes were then added to complete RPMI-1640 to transfect INS-1E cells. Transfection efficiency was estimated by fluorescent microscopy of GFP.

Western Blot analysis

Human islets or INS-1E cells were washed three times with ice-cold PBS after medium removal and lysed with RIPA lysis buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Protease and Phosphatase Inhibitors (Thermo Fisher Scientific (TFS), MA, USA). Samples went under multiple freeze-thaw cycles and finally incubated on ice for 30 minutes with intermittent vortexing. The cell lysates were centrifuged at 16000 x g for 20 minutes at 4°C and the clear supernatant containing the extracted proteins were kept at -80°C for storage. Protein concentrations were measured by the BCA protein assay (TFS). Equivalent amounts of protein from each condition were run on a NuPAGE 4-12% Bis-Tris gel (Invitrogen; CA, USA) and electrically transferred into PVDF membranes. Membranes were blocked at RT using mixture of 2.5% milk (Cell Signaling Technology/CST, MA, USA) and 2.5% BSA (SERVA Electrophoresis GmbH, Heidelberg, Germany) for 1h and incubated overnight at 4°C with rabbit anti-cleaved caspase-3 (#9664), rabbit anti-YAP(D8H1X; #14074), rabbit anti-MST1 (#3682), rabbit anti-GAPDH (#2118), rabbit anti-β-actin (#4967; all CST), and mouse anti-Enterovirus/VP1 (clone 5-D8/1 #M7064, Dako). All primary antibodies were used at 1:1,000 dilution in 1xTris-buffered saline plus Tween-20 (1xTBS-T) containing 5% BSA and 0.5% NaN_3. Later, membranes were incubated with horseradish-peroxidase-linked anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch, PA, USA) and developed using Immobilon Western chemiluminescence assay system (Millipore, MA, USA). Analysis of the immunoblots was performed using Vision Works LS Image Acquisition and Analysis software Version 6.8 (UVP BioImaging Systems, CA, USA).

Measurement of CXCL10 release

CXCL10 secretion into culture media from controls and virus infected isolated human islets was measured by Human CXCL10/IP-10 DuoSet ELISA kit (#DY266-05, R&D Systems, MN, USA) according to the manufacturer's instructions.

qPCR analysis

Total RNA was isolated from cultured isolated islets or INS-1E/PANC-1 cells using TriFast (PEQLAB Biotechnologie, Germany). 500-1000ng of RNA were reverse transcribed to cDNA (RevertAid reverse transcriptase, Thermo Fisher Scientific (TFS), MA, USA). Quantitative RT-PCR was carried out as previously described [20] using Biosystems StepOne Real-Time PCR system (Applied Biosystems, CA, USA) with TaqMan assays or SybrGreen (Applied Biosystems). TaqMan® Gene Expression Assays were used for Stk4 (#Hs00178979), CTGF (#Hs01026927-g1), CXCL10 (#Hs00171042), IFNB1 (#Hs02621180), OAS1 (#Hs00973637), DDX58 (#Hs01061436), TLR3 (#Hs01551078), IFIH1 (#Hs00223420), ACTB (#Hs99999903), Stk4 (#Mm00451755), Tuba1a (#Mm00846967), Stk4 (#Rn01750112), and ACTB (#Rn00667869). EV-RNA was detected by using a SybrGreen primer pair (forward: 5′-CGGCCCCTGAATGCGGCTAA-3′; reverse: 5′-GAAACACGGACACCCAAAGTA-3′). The relative changes in gene expression were analyzed by ΔΔCT method.

Chromatin immunoprecipitation (ChIP) assay

4×10^6 INS-1E cells were dual-cross-linked consecutively with 2mM disuccinimidyl glutarate (DSG, #20593, TFS) for 45 min and 1% formaldehyde for 10 min. ChIP was performed according to the user's instructions for SimpleChIP Enzymatic Chromatin IP Kit (#9003, CST). In brief, chromatin DNA was digested with micrococcal nuclease (MNase). Immunoprecipitation reactions were carried out with chromatin extracts using IgG negative control or YAP antibodies (both CST) overnight at 4°C. Proteinase K was added for de-crosslinking, and samples were incubated for 4 h in a water bath at 65°C. Precipitated DNA was quantitated by real-time PCR analysis. The SybrGreen primers used in this study to amplify the promoter regions were: STK4#1 fw 5´ CCTCGACTTCCTCATGGCTG 3´, rev 5´ ACTAGGGACCCAATGAGCCT 3´; STK4#2 fw 5´ GCCAGCCTGTTTCTTCCTCT 3´, rev 5´ CTCCACGACTGGTGAGGTTT 3´; ANKRD1 fw 5´ GTGTGATGCACAATGCTTGC 3´, rev 5´CTTATCGGGAAGCCAGGGAC 3´. ANRD1, a YAP target gene, was used as a positive control. All ChIP signals were expressed as a fold enrichment (as a ratio of the YAP signal to the IgG signal for each respective condition).

Dual reporter assay

Hela cells were seeded into 6-well plates and transiently transfected with pEZX-PG04.1 reporter construct (#RPRM55953-PG04, Genecopoiea, MD, USA) using jetPRIME® transfection reagent. After 48h post-transfection, stable Hela cells expressing conditional Gaussia Luciferase (GLuc) reporter located downstream of rat *STK4* promoter and constitutive Secreted Alkaline Phosphatase (SEAP) was generated by puromycin sections. After selection, Hela cells were maintained in culture medium containing 2μg/ml puromycin. Hela stable cells were then transduced with Ad-LacZ or Ad-YAP treated with or without VP. After 48h, medium was analyzed for activities of both GLuc and SEAP using the Secrete-PairTM Dual Luminescence and Gaussia Luciferase Assay Kit (Genecopoiea) per manufacturer's

instructions. The data are presented as the relative change in normalized GLuc activities to SEAP.

Immunofluorescence

Paraffin-embedded bouin-fixed human islets or human primary pancreatic cells were deparaffinized and rehydrated. INS-1E or PANC-1 cells were fixed with 4% PFA for 30 min followed by 4 min permeabilization with 0.5 % Triton-X-100. Fixed or embedded cells were then blocked with blocking buffer containing 3% BSA and then incubated overnight at 4°C with the following antibodies (single or double): guinea pig anti-insulin (#IR002, FLEX polyclonal DAKO), mouse, mouse anti-Enterovirus/VP1 (clone 5-D8/1 #M7064, Dako), mouse anti-chromogranin (#ab715, Abcam) and mouse anti-cytokeratin 19/CK-19 (#15463-1, Abcam). The next day sections were incubated with Cy3-conjugated donkey anti-mouse (#715-165-150), FITC-conjugated donkey anti-guinea pig (706-096-148) or FITC- conjugated donkey anti-mouse (#715-095-150) secondary antibodies (all from Jackson Immuno Research Laboratories, West Grove, PA; 1:100 dilution) for 1h at RT or 37°C. β-cell apoptosis in fixed human islet sections were performed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique according to the manufacturer's instructions (In Situ Cell Death Detection Kit, TMR red; Roche) and double stained for insulin. Slides were mounted with Vectashield with 4′6-diamidino-2-phenylindole (DAPI, #H-1200-10, Vector Labs).

YAP immunohistochemistry

Detection of YAP protein in pancreatic tissue was carried out by classical immunohistochemistry (IHC) coupled with SuperBoost™ tyramide signal amplification (#B40931, Biotin XX Tyramide SuperBoost™ Kit, Streptavidin, TFS). After tissues deparaffinization and dehydration, endogenous peroxidase was quenched by 3% hydrogen peroxidase for 1h at RT. Tissues were blocked by applying the blocking buffer for 1h at RT and subsequently were incubated with rabbit anti-YAP (D8H1X, #14074, CST) antibody alone or in combination with mouse anti-chromogranin (#ab715, Abcam) antibody overnight. A day after, sections were washed with PBS and were incubated with rabbit poly-HRP-conjugated secondary antibody for 1h at RT. To amplify the signal, a Tyramide working solution was prepared according to the manufacturer's instructions by adding the Tyramide solution and hydrogen peroxide into the reaction buffer. Sections were incubated for 10min at RT followed by applying reaction stop regent for 3min. The chromogenic detection was completed by applying ABC (Avidin/Biotin) system (VECTASTAIN® ABC-HRP Kit, Peroxidase-Standard, #PK-4000) for 1h and DAB substrate (3,3'-diaminobenzidine-DAB Substrate Kit, Peroxidase-HRP, #SK-4100, all Vector Laboratories) for 5 min; both at RT. For the YAP-chromogranin double labeling, staining continued by using fluorescein isothiocyanate (FITC)-conjugated secondary donkey anti-mouse antibody (#715-095-150, Jackson Immuno Research

Laboratories, West Grove, PA) for 1h at RT. Counterstaining was performed by either DAPI or Hematoxylin.

RNAscope mRNA in situ hybridization assay

YAP or YAP/CVB3-4 double staining was performed using the RNAscope 2.5 HD Detection Duplex Reagent RNAscope kit (#322430, Advanced Cell Diagnostics) according to the manufacturer's instructions. Human Yap1 (#419131-C2; ACD), and human CVB (#409301, V-CVB4; #409291, V-CVB3) probes were used to detect Yap1 or CVB mRNAs. Briefly, tissue sections were incubated for 1 h at 60 °C, deparaffinized and rehydrated by xylene and 100% ethanol for 10 and 2min, respectively. Target retrieval was performed for 15 min at 95-97 °C, followed by protease treatment for 15 min at 40 °C. Probes were then hybridized for 2h at 40 °C followed by repeated washing with wash buffer and then kept in 5x Saline-sodium citrate (SSC) buffer overnight. RNAscope amplification was carried out using two independent signal amplification systems based on HRP and AP labeled probes and ultimately visualized by red and green chromogenic substrates. At the end, sections were counterstained with Hematoxylin.

Single molecule fluorescence in situ hybridization (smFISH)

smFISH was used to detect enterovirus mRNA in pancreatic tissue sections by using single-molecule oligonucleotide probes carried out according to the highly sensitive protocol that was previously established in our lab [86]. FISH Probes were synthesized by Stellaris® (Biosearch Technologies, Inc.; Petaluma, CA, USA), and labeled with Quasar 570 [8, 33]. The three probes sets recognizes various enteroviral strains for positive strand enteroviral RNA based on sequence similarities. FFPE sections were deparaffinized with Xylene for 30 min at 70°C and 10 min at room temperature then rehydrated in 100, 95, and 70% ethanol for 20, 10, and a minimum 60 min respectively. Sections were covered with 0.2M HCL for 20 min followed by washing in prewarmed 2xSSC for 15min in a shaking water bath at 70°C. For antigen retrieval, pepsin was used for 10min in 37°C humidified chamber and washed two times with PBS. Before hybridization, samples were equilibrated 2 times with buffer made by 10% formamide and 2XSSC. Probes hybridized overnight at 37 °C. Next day slides underwent several times of washing at 37°C in a shaking water bath including 2xSSC plus 10% formamide for 40 min, 2xSSC 30 min, 1xSSC 30 min, 0.1xSSC for 20 min. Thereafter, classical immunostaining was performed for YAP and DAPI as detailed above. A 60x oil-immersion objective was used to acquire images by a Nikon Ti MEA53200 (NIKON GmbH, Düsseldorf, Germany) microscope.

Image analysis and quantification

Images were obtained using an inverse Nikon Ti2-A MEA54100 (NIKON GmbH, Düsseldorf, Germany) microscope with NIS-Elements Software (BR-ML). To quantify the YAP-positive area in the human exocrine pancreas, 229 different fields (in dependent positions) from 13 control donors, 223 from 15 AAb^+ donors, and 284 from 15 T1D donors were analyzed for YAP

intensity by Image J.JS (v0 5.6) and data presented as % of YAP-positive area. The YAP-positive fraction in the islet was quantified manually by counting the number of YAP-positive cells in the pancreatic islet normalized to the number of all chromogranin-positive cells in the pancreas. % double YAP/chromogranin-positive cells were quantified by the number of double positive cells normalized to the number of chromogranin-positive cells. The infection rate in INS-1E cells was calculated by counting the number of VP1-positive cells divided by all cells from 40-50 randomly captured images under the 60x objective throughout the well. In PANC-1 cells, the same analysis was carried out with 12 randomly captured images under the 20x objective. Total number of cells in each image was quantified by manually counting all DAPI-stained nuclei using NIS-elements and used for normalization and to calculate the percentage of VP1-positive cells in the respective images. To quantify YAP-CVB3/4 double positive cells from RNAScope, infected cells were classified into two categories, low or single infection (5-10 puncta/cell) and full or cluster infection (>10 puncta/cell). Neighboring YAP-positive cells were the cells located exactly next to the infected cell. RNAScope YAP-mRNA was quantified by counting cells with YAP^+ puncta normalized to the number of all nuclei. Apoptosis and infection in isolated human islets were quantified by double-positive TUNEL/insulin or VP1/insulin cells normalized to all insulin-positive cells for each islet.

Statistical analyses

All statistics were performed using GraphPad Prism software (GraphPad Software Inc.). Gaussian distribution was determined by using a D'Agostino-Pearson normality test. Statistical comparisons between groups were analyzed for significance by a paired or unpaired two-tailed Student's t-test and a one-way or two-way analysis of variance (ANOVA) with Holm-Sidak multiple comparisons correction for a parametric test, or a Kruskall-Wallis test followed by Dunn post-test correction for a nonparametric test. A Spearman correlation analysis was used to assess the correlation between YAP protein expression and other markers. P value<0.05 was considered statistically significant. Data are presented as means ± SEM. The exact values of *n* (refers to number of donors or mice, or number of independent biological experiments or independent measurements/positions), and statistical significance are reported in the figure legends.

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Figure legends

Figure 1. YAP is highly upregulated in pancreas of T1D and AAb⁺ organ donors. YAP protein and *Yap1* mRNA labeling were analyzed in FFPE sections of pancreases from 13 control and 15 AAb⁺ organ donors without diabetes and 15 donors with T1D from the nPOD pancreas collection. **(A,B)** Representative images from different donors **(A)** and quantification **(B)** of the percentage of YAP⁺ area in the exocrine pancreas from FFPE sections of control donors without diabetes (n=229 independent positions throughout 13 pancreas slides from 13 donors), donors without diabetes but expressing T1D-associated autoantibodies (AAb⁺) (n=223 independent positions from 15 donors), and donors with T1D (n=284 independent positions from 15 donors). **(C,D)** Representative images **(C)** and quantification **(D)** of the percentage of YAP⁺ cells within islets of controls (n=10), AAb⁺ (n=10), and donors with T1D (n=15) of the number of islet cells. **(E,F)** Representative images **(E)** and quantification **(F)** of YAP (brown), and late endocrine marker chromogranin (green) double-positive cells from controls (n=3; 16671 islet cells), AAb⁺ donors (n=3; 14237 islet cells), and donors with T1D (n=6; 15116 islet cells). **(G,H)** Representative images **(G)** and quantification **(H)** of *Yap1* mRNA (pink) by RNAscope in situ hybridization of controls (n=30 independent positions from 3 donors), AAb⁺ donors (n=30 independent positions from 3 donors), and donors with T1D (n=33 independent positions from 3 donors). **(I)** Association of YAP protein expression between endocrine islets and exocrine pancreas in AAb⁺ (n=10; grey circles) and in donors with T1D (n=15; black circles). Box plots showing single analytes and median (min to max). **(A,C,G)** sections were counterstained with Hematoxylin. Data are expressed as means ± SEM. *p<0.05, **p<0.001, ***p<0.0001; by Kruskall-Wallis test followed by Dunn post-test correction for B, and one-way ANOVA with Holm-Sidak multiple comparisons correction for D,F and H. Scale bars depict 50 µm (A,C,G-upper panel) and 10 µm (E,G-lower panel).

Figure 2. YAP colocalizes and correlates with enteroviral RNA expression in the pancreas

(A-D) Detection and quantification of *Yap1* mRNA (pink) and viral RNA-CVB3/4 (turquois) by RNAscope in situ hybridization from FFPE nPOD pancreas sections of AAb⁺ donors (n=9) and donors with T1D (n=10). **(A)** Representative images of *Yap1*/CVB-RNA double labelling from AAb⁺ and T1D pancreatic sections **(B,C)** and total distribution and quantification throughout

the whole pancreas section differentiated in YAP-viral RNA double positive cells (YAP^+/CVB^+; purple) CVB-positive cells in close proximity of YAP-positive neighbor cells (n-YAP^+/CVB^+; blue) or YAP-negative but CVB-RNA-positive cells (YAP^-/CVB^+; gray). **(D)** Quantification of all viral RNA-positive cells throughout the whole pancreas section in AAb^+ and T1D donors presented as the mean number of single (white; 5-10 single puncta/cell) or cluster (black; >10 single puncta/cell) infected cells. **(E;F)** Association between YAP protein expression and number of enterovirus-positive cells by smFISH for enteroviral RNA detection in AAb^+ donors (n=9) and donors with T1D (n=10). **(F)** Representative microscopical images of enteroviral RNA (red; Stellaris probes) and YAP protein (brown; IHC) expression in the pancreas showing YAP^+/Enterovirus$^+$ cells (YAP^+/V^+) and enteroviral positive cells in close proximity of YAP-positive neighbor cells (n-YAP^+/V^+). Data are expressed as means ± SEM. *p<0.05, **p<0.01, ***p<0.001; by one-way ANOVA with Holm-Sidak multiple comparisons correction for C, and two-tailed unpaired Student *t*-test for D. Scale bars depict 10 µm.

Figure 3. YAP enhances coxsackievirus replication and potentiates coxsackievirus-induced islet inflammation and β-cell apoptosis. (A,C,D,K,L) INS-1E cells and **(B,E-H,M-W)** human pancreatic islets transduced with Ad-YAP or Ad-LacZ control and then infected with CVB4 (MOI=5) for 24h (INS-1E) or CVB3 and -4 (MOI=10) for 48h (human islets). **(A,B)** Intracellular CVB3 or -4 RNA genome of **(A)** INS-1E cells (n=3 independent experiments) and **(B)** human pancreatic islets (n=4 organ donors). **(C-F)** Representative Western blots and pooled quantitative densitometry analysis of VP1 in **(C,D)** INS-1E cells (n=7 independent experiments) and **(E,F)** human islets (n=6 organ donors). **(G,H)** Representative images **(G)** and quantitative percentage of VP1-positive β-cells **(H)** are shown (n=4 organ donors). **(I,J)** Human primary pancreatic cells transduced infected with CVB3 and -4 (MOI=10) for 48h treated with or without 2.5 uM verteporfin (VP) for the last 24h. Representative images **(I)** and quantitative percentage of CK19-positive ductal cells **(J)** are shown (n=6 independent positions from two organ donors). **(K-N)** Representative Western blots and pooled quantitative densitometry analysis of cleaved caspase 3 in **(K,L)** INS-1E cells (n=7 independent experiments) and **(M,N)** human islets (n=6 organ donors). **(O,P)** Representative images **(O)** and quantitative percentage of TUNEL-positive β-cells **(P)** are shown (n=3 organ donors). **(Q-V)** qPCR for **(Q)** IFNB1, **(R)** CXCL10, **(S)** OSA1, **(T)** IFIH1, **(U)** DDX58, and **(V)** TLR3 mRNA expression in isolated human islets normalized to actin (n=3-5 organ donors). **(W)** Secreted CXCL10 analyzed by ELISA in the culture media (n=8 independent samples from five organ donors). Data are expressed as means ± SEM. *p<0.05, **p<0.01, ***p<0.001; by two-tailed paired (A,B,D,F,H,J,L,N,P,W) or ratio paired (Q-V) Student *t*-test. Scale bars depict 50 µm (G,O) and 10 µm (I).

Figure 4. A YAP-TEAD-MST1 feedback loop controls CVB replication and cell death. (A,B,E) INS-1E cells and **(C,D)** human pancreatic islets transduced with Ad-YAP or Ad-LacZ

control for 48h. **(A-D)** Representative Western blot and pooled quantitative densitometry analysis of MST1 in **(A,B)** INS-1E cells (n=6 independent experiments) and **(C,D)** human islets (n=7 organ donors). **(E)** qPCR for *STK4* mRNA expression in INS-1E cells normalized to actin (n=3 independent experiments). **(F-I)** INS-1E cells and human islets transduced with Ad-YAP or Ad-LacZ control for 48h treated with or without 1-5 uM verteporfin (VP) for last 6h (INS-1E) or 24h (human islets). Western blots and pooled quantitative densitometry analysis of MST1 in **(F,G)** INS-1E cells (n=3 independent experiments) and **(H,I)** human islets (n=3 organ donors). **(K-N)** Hela cells or INS-1E cells transduced with Ad-YAP or Ad-LacZ control for 48h treated with or without 1 uM verteporfin (VP) for the last 24h. **(K)** Hela cells culture media was analyzed for activities of both GLuc and SEAP and data presented as the relative change in normalized GLuc to SEAP. **(L-N)** ChIP from INS-1E cells was performed with control IgG, or YAP antibody as indicated. The presence of **(L,M)** *STK4* and **(N)** *ANKRD1* promoters was detected by PCR. Data presented as fold enrichment in which ChIP signals are divided by the IgG-antibody signals, representing the fold increase in signal relative to the background signal. **(O-S)** INS-1E cells transfected with siMST1 or control siScr and then transduced with Ad-YAP or Ad-LacZ control for 48h. All cells were infected with CVB4 (MOI=5) for last 24h. **(O,P)** Representative Western blot and pooled quantitative densitometry analysis of MST1, VP1 and cleaved caspase 3 in INS-1E cells (n=3 independent experiments). **(Q,R)** Representative images **(Q)** and quantitative percentage of VP1-positive cells **(R)** are shown (n=28-30 independent positions). **(S)** Intracellular CVB4 RNA genome of INS-1E cells (n=4 independent experiments). Data are expressed as means ± SEM. *p<0.05, **p<0.01, ***p<0.001; by one-way and two-way ANOVA with Holm-Sidak multiple comparisons correction for K,L,M,N,P,R and F,H,J respectively, and two-tailed paired Student *t*-test for B,D,E. Scale bar depicts 10 µm.

Figure S1. YAP is elevated in pancreases of T1D and AAb$^+$ organ donors. (A,B) Representative images from FFPE pancreas sections of control donors without diabetes, donors without diabetes but expressing T1D-associated autoantibodies (AAb$^+$), and donors with T1D of **(A)** YAP in exocrine pancreas and **(B)** of YAP (brown) and chromogranin (green) in the islet area. **(C-E)** Association between YAP protein expression in pancreases and **(C)** age and **(D)** BMI in AAb$^+$ donors (n=15, grey) and donors with T1D (n=15, black) and **(E)** with HbA1C in AAb$^+$ donors (n=12) and donors with T1D (n=13). Scale bars depict 50 µm (A) and 10 µm (B).

Figure S2. Various categories of infected pancreatic cells. Representative images of single (upper panel; 5-10 puncta of CVB-RNA expression/cell) and cluster (lower panel, >10 puncta of CVB-RNA expression/cell) infections of three different categories of YAP-viral RNA double positive cells (YAP$^+$/CVB$^+$), CVB-positive cells in close proximity of YAP-positive neighbor cells (n-YAP$^+$/CVB$^+$) or YAP-negative but CVB-RNA-positive cells (YAP$^-$/CVB$^+$) in human pancreases from AAb$^+$ and T1D donors. Scale bar depicts 10 µm.

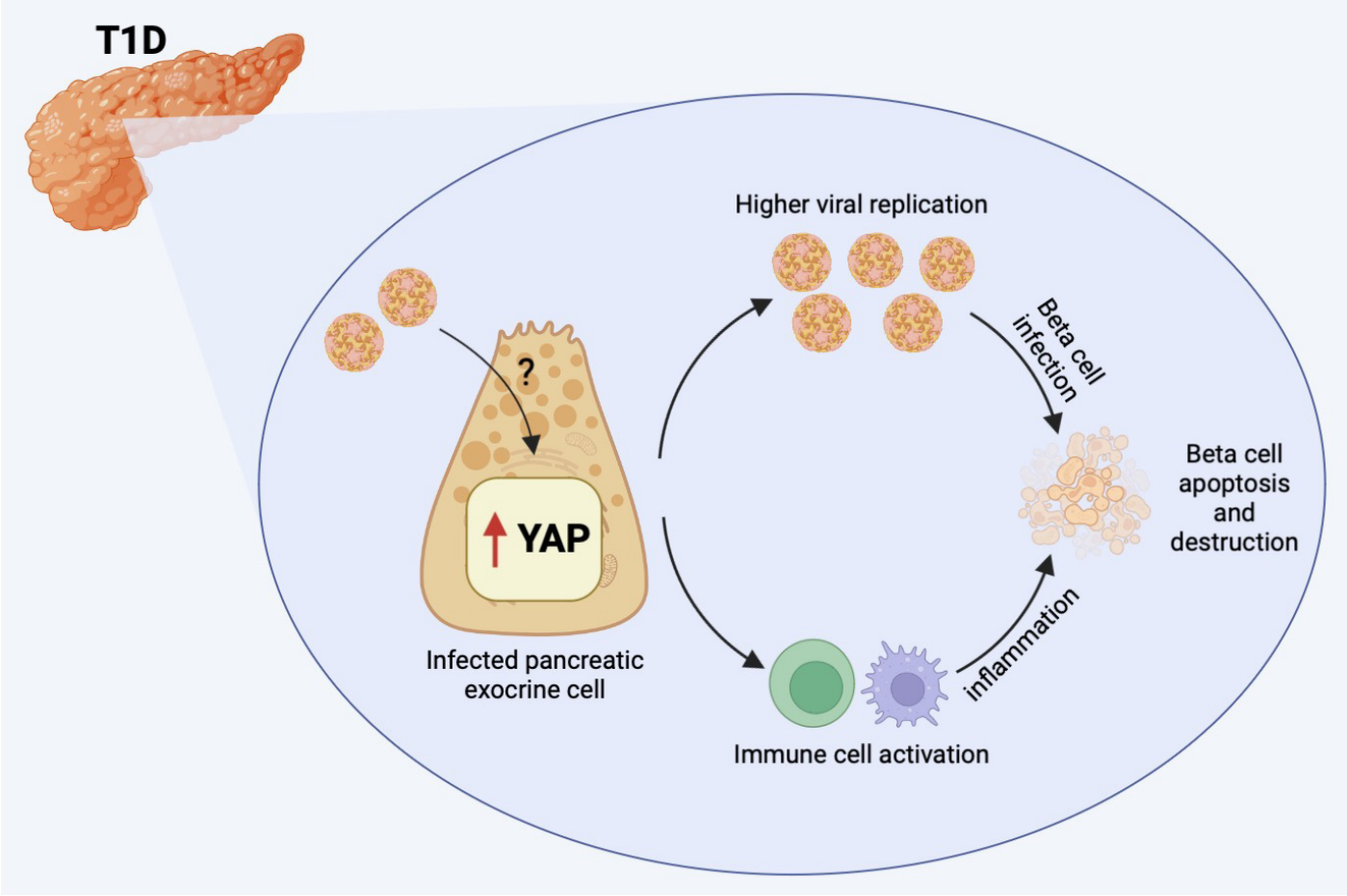
Figure S3. YAP regulates coxsackieviruses replication and β-cell apoptosis. (A) Representative image of triple VP1-, YAP-, and insulin-positive β-cells is shown (n=4 organ donors). **(B-E)** PANC-1 cells infected with CVB4 for 48h treated with or without 0-5 μM verteporfin (VP) for last 24h. **(B)** Intracellular CVB4 RNA genome of PANC1 cells (n=3 independent experiments). **(C)** qPCR for CTGF mRNA expression in PANC1 cells (n=3 independent experiments). **(D,E)** Representative images **(D)** and quantitative percentage of VP1-positive cells **(E)** are shown (n=12 independent positions). **(F,G)** INS-1E cells transduced with Ad-YAP or Ad-LacZ control and then treated with Poly I:C for 24h. **(F)** Representative Western blot and **(G)** pooled quantitative densitometry analysis of cl caspase 3 in INS-1E cells (n=3 independent experiments). **(H-M)** qPCR for **(H)** *IFNB1*, **(I)** *CXCL10*, **(J)** *OSA1*, **(K)** *IFIH1*, **(L)** *DDX58*, and **(M)** *TLR3* mRNA expression in isolated human islets normalized to actin (n=3-5 organ donors). Data are expressed as means ± SEM. *p<0.05, **p<0.01, ***p<0.001; by one-way ANOVA with Holm-Sidak multiple comparisons correction for B and C, by two-tailed unpaired Student *t*-test for E, and by two-tailed paired (G) or ratio paired (H-M) Student *t*-test. Scale bars depict 10 μm (A) and 50 μm (D).

Figure S4. YAP-TEAD-MST1 negative feedback loop controls CVB replication and associated cell death. (A) qPCR for *STK4* mRNA expression in islets isolated from β-YAP and control mice recovered after isolation overnight (n=8-9 mice). **(B,C)** INS-1E cells and **(D,E)** human pancreatic islets infected with CVB4 (MOI=5) for 24h (INS-1E) or CVB4 (MOI=10) for 48h (human islets). **(B)** Representative Western blot and **(C)** pooled quantitative densitometry analysis of MST1 in INS-1E cells (n=5 independent experiments). **(D)** Representative Western blot and **(E)** pooled quantitative densitometry analysis of MST1 in human islets (n=5 organ donors). **(F)** A schematic which presents complementary approaches to block YAP/TEAD signaling. **(G-I)** INS-1E cells transfected with GFP, active YAP or YAP-S94A constructs for 48h. **(G)** qPCR for STK4 mRNA expression in INS-1E cells (n=3 independent experiments). **(H)** Representative Western blot and **(I)** pooled quantitative densitometry analysis of MST1 in INS-1E cells (n=4 independent experiments). **(J-L)** INS-1E cells transfected with GFP, or TEADi constructs and then transduced with Ad-YAP or Ad-LacZ control for 48h. **(J)** qPCR for STK4 mRNA expression in INS-1E cells (n=5 independent experiments). **(K)** Representative Western blot and **(L)** pooled quantitative densitometry analysis of MST1 in INS-1E cells (n=4 independent experiments). **(M)** Predicted TEAD1 binding sites in the rat *STK4* promoter identified by EPD (https://epd.epfl.ch/index.php). **(N-R)** INS-1E cells transfected with MST1-K59 or control GFP constructs and then transduced with Ad-YAP or Ad-LacZ control for 48h. All cells were infected with CVB4 (MOI=5) for last 24h. **(N,O)** Representative Western blot **(N)** and pooled quantitative densitometry analysis **(O)** of MST1, VP1 and cleaved caspase 3 in INS-1E cells (n=3 independent experiments). **(P,Q)** Representative images **(P)** and quantitative percentage of VP1-positive cells **(Q)** are shown (n=40-50 independent positions).

(R) Intracellular CVB4 RNA genome of INS-1E cells (n=3 independent experiments). Data are expressed as means ± SEM. *p<0.05, **p<0.01, ***p<0.001; by one-way ANOVA with Holm-Sidak multiple comparisons correction for G and I, by Kruskall-Wallis test followed by Dunn post-test correction for Q, by two-tailed unpaired Student *t*-test for A,L and by two-tailed paired Student *t*-test for C,E,J,O,R. Scale bar depicts 10 µm.

Figure S5. Our model how a vicious cycle of YAP expression and CVB replication in the human pancreas may lead to T1D.

YAP is highly elevated in the pancreas of patients with T1D; it boosts enteroviral replication, induces a strong IFN response and islet inflammation ultimately leading to β-cell apoptosis and destruction. At the molecular level, YAP, through a feedback mechanism, induces the expression of its own negative regulator MST1 limiting YAP-driven viral replication and induces apoptosis of infected cells. Viruses and a local inflammatory milieu remain in the pancreas and T1D develops.


T1D
Higher viral replication
Beta cell infection
Beta cell apoptosis and destruction
?
YAP
Infected pancreatic exocrine cell
inflammation
Immune cell activation

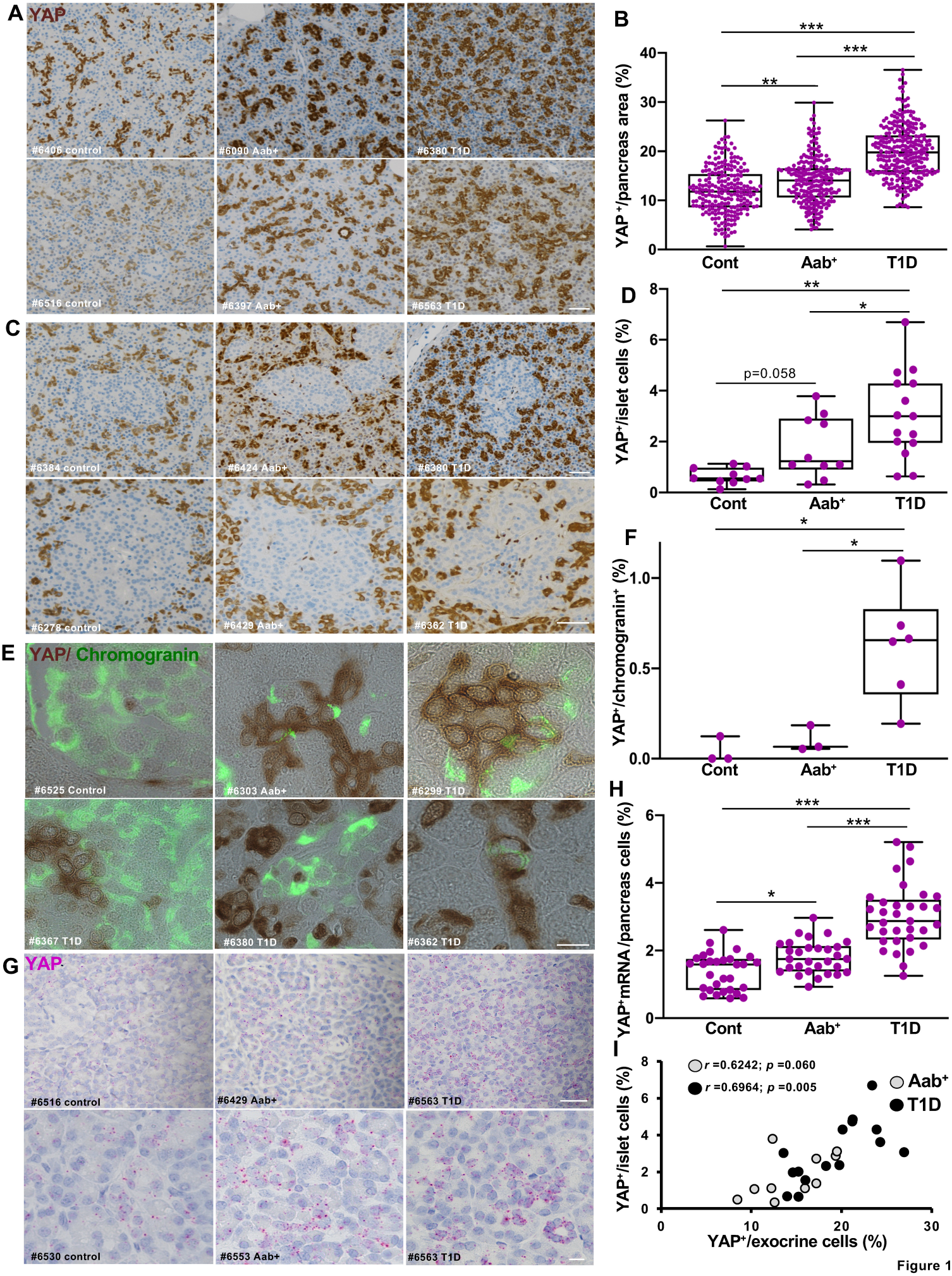

A
YAP
#6406 control
#6090 Aab+
#6380 T1D
#6516 control
#6397 Aab+
#6563 T1D
C
#6384 control
#6424 Aab+
#6380 T1D
#6278 control
#6429 Aab+
#6362 T1D
E
YAP/ Chromogranin
#6525 Control
#6303 Aab+
#6299 T1D
#6367 T1D
#6380 T1D
#6362 T1D
G
YAP
#6516 control
#6429 Aab+
#6563 T1D
#6530 control
#6553 Aab+
#6563 T1D
B
YAP+/pancreas area (%)
Cont
Aab+
T1D
D
YAP+/islet cells (%)
p=0.058
F
YAP+/chromogranin+ (%)
H
YAP+mRNA/pancreas cells (%)
I
YAP+/islet cells (%)
YAP+/exocrine cells (%)
r =0.6242; p =0.060
r =0.6964; p =0.005
Aab+
T1D

Figure 1

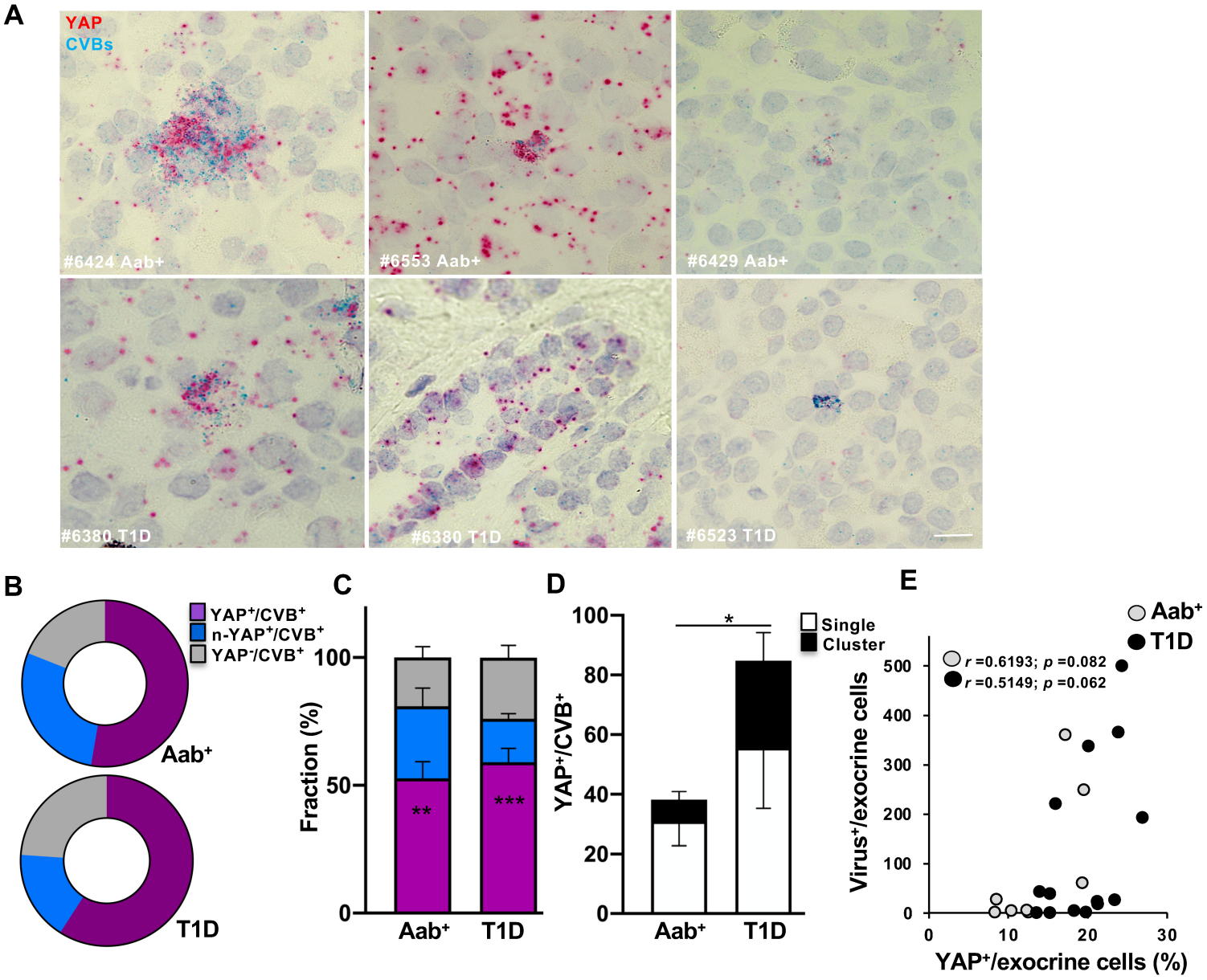

A
YAP
CVBs
#6424 Aab+
#6553 Aab+
#6429 Aab+
#6380 T1D
#6380 T1D
#6523 T1D
B
YAP+/CVB+
n-YAP+/CVB+
YAP-/CVB+
Aab+
T1D
C
Fraction (%)
100
50
0
**
***
Aab+
T1D
D
YAP+/CVB+
100
80
60
40
20
0
*
Single
Cluster
Aab+
T1D
E
Aab+
T1D
r =0.6193; p =0.082
r =0.5149; p =0.062
Virus+/exocrine cells
500
400
300
200
100
0
0
10
20
30
YAP+/exocrine cells (%)
F
YAP
enteroviral RNA
#6429 Aab+
#6424 Aab+
#6347 Aab+
YAP+/V+
n-YAP+/V+
#6380 T1D
#6396 T1D
#8380 T1D

Figure 2

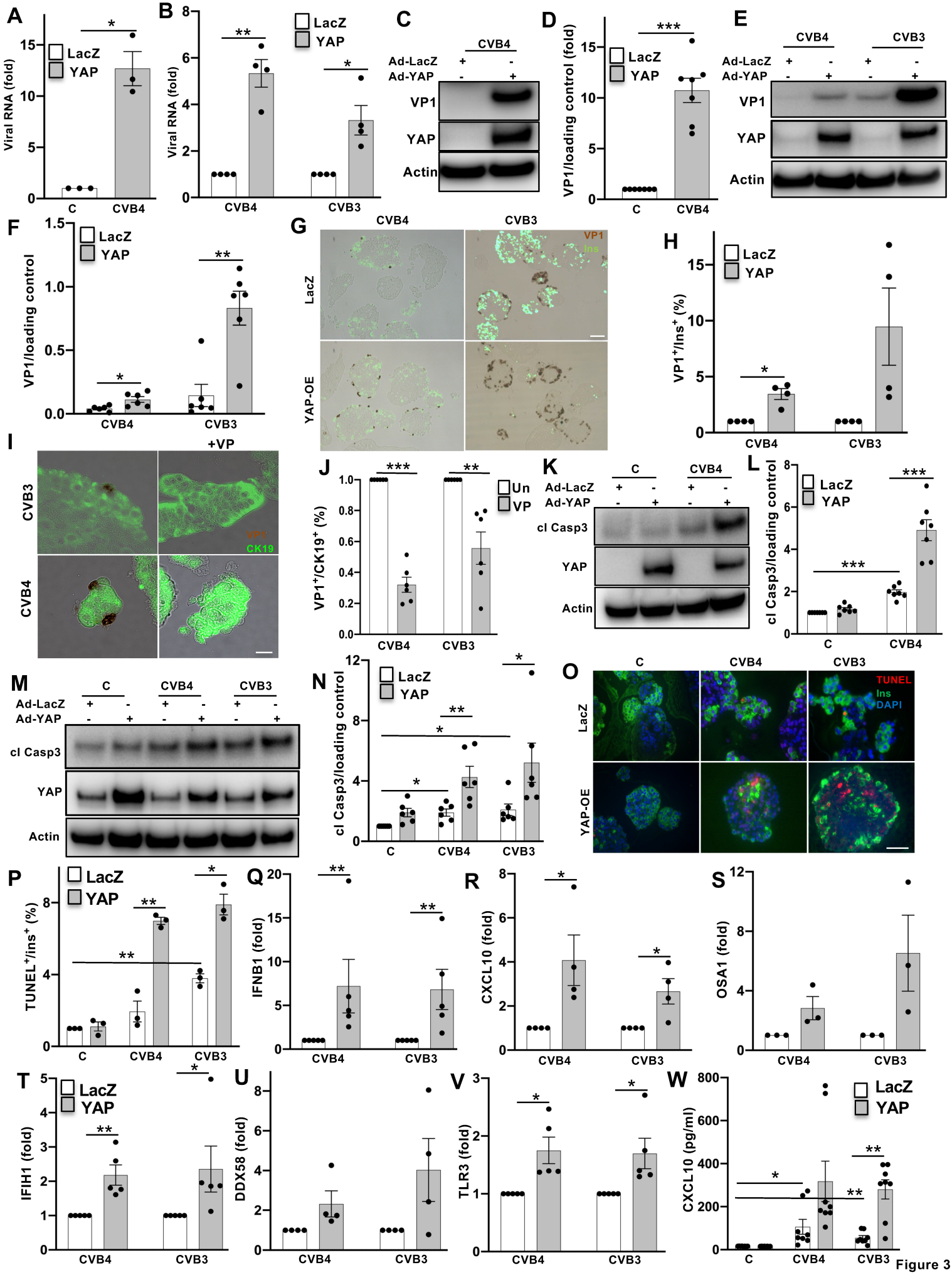

A
Viral RNA (fold)
LacZ
YAP
C
CVB4
B
Viral RNA (fold)
LacZ
YAP
CVB4
CVB3
C
CVB4
Ad-LacZ
Ad-YAP
VP1
YAP
Actin
D
VP1/loading control (fold)
LacZ
YAP
C
CVB4
E
CVB4
CVB3
Ad-LacZ
Ad-YAP
VP1
YAP
Actin
F
VP1/loading control
LacZ
YAP
CVB4
CVB3
G
CVB4
CVB3
LacZ
YAP-OE
VP1
Ins
H
VP1+/Ins+ (%)
LacZ
YAP
CVB4
CVB3
I
+VP
CVB3
CVB4
VP1
CK19
J
VP1+/CK19+ (%)
Un
VP
CVB4
CVB3
K
C
CVB4
Ad-LacZ
Ad-YAP
cl Casp3
YAP
Actin
L
cl Casp3/loading control
LacZ
YAP
C
CVB4
M
C
CVB4
CVB3
Ad-LacZ
Ad-YAP
cl Casp3
YAP
Actin
N
cl Casp3/loading control
LacZ
YAP
C
CVB4
CVB3
O
C
CVB4
CVB3
LacZ
YAP-OE
TUNEL
Ins
DAPI
P
TUNEL+/ins+ (%)
LacZ
YAP
C
CVB4
CVB3
Q
IFNB1 (fold)
CVB4
CVB3
R
CXCL10 (fold)
CVB4
CVB3
S
OSA1 (fold)
CVB4
CVB3
T
IFIH1 (fold)
LacZ
YAP
CVB4
CVB3
U
DDX58 (fold)
CVB4
CVB3
V
TLR3 (fold)
CVB4
CVB3
W
CXCL10 (pg/ml)
LacZ
YAP
C
CVB4
CVB3

Figure 3

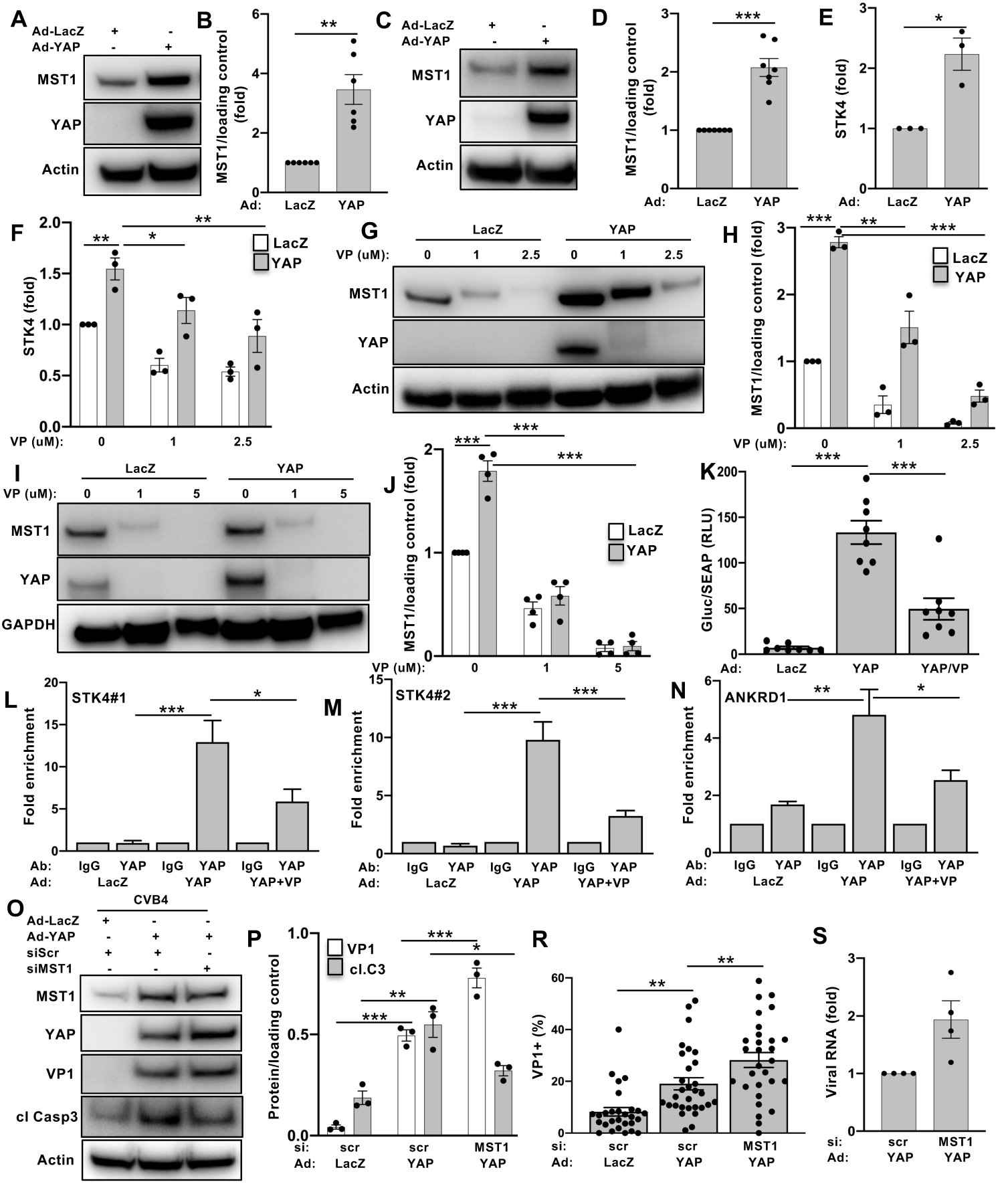

A
Ad-LacZ + -
Ad-YAP - +
MST1
YAP
Actin
B
MST1/loading control (fold)
Ad: LacZ YAP
C
Ad-LacZ + -
Ad-YAP - +
MST1
YAP
Actin
D
MST1/loading control (fold)
Ad: LacZ YAP
E
STK4 (fold)
Ad: LacZ YAP
F
STK4 (fold)
LacZ
YAP
VP (uM): 0 1 2.5
G
LacZ YAP
VP (uM): 0 1 2.5 0 1 2.5
MST1
YAP
Actin
H
MST1/loading control (fold)
LacZ
YAP
VP (uM): 0 1 2.5
I
LacZ YAP
VP (uM): 0 1 5 0 1 5
MST1
YAP
GAPDH
J
MST1/loading control (fold)
LacZ
YAP
VP (uM): 0 1 5
K
Gluc/SEAP (RLU)
Ad: LacZ YAP YAP/VP
L
STK4#1
Fold enrichment
Ab: IgG YAP IgG YAP IgG YAP
Ad: LacZ YAP YAP+VP
M
STK4#2
Fold enrichment
Ab: IgG YAP IgG YAP IgG YAP
Ad: LacZ YAP YAP+VP
N
ANKRD1
Fold enrichment
Ab: IgG YAP IgG YAP IgG YAP
Ad: LacZ YAP YAP+VP
O
CVB4
Ad-LacZ + - -
Ad-YAP - + +
siScr + + -
siMST1 - - +
MST1
YAP
VP1
cl Casp3
Actin
P
Protein/loading control
VP1
cl.C3
si: scr scr MST1
Ad: LacZ YAP YAP
R
VP1+ (%)
si: scr scr MST1
Ad: LacZ YAP YAP
S
Viral RNA (fold)
si: scr MST1
Ad: YAP YAP
Q
CVB/siScr/LacZ
CVB/siScr/YAP
CVB/siMST1/YAP
VP1
Ins
DAPI

Figure 4

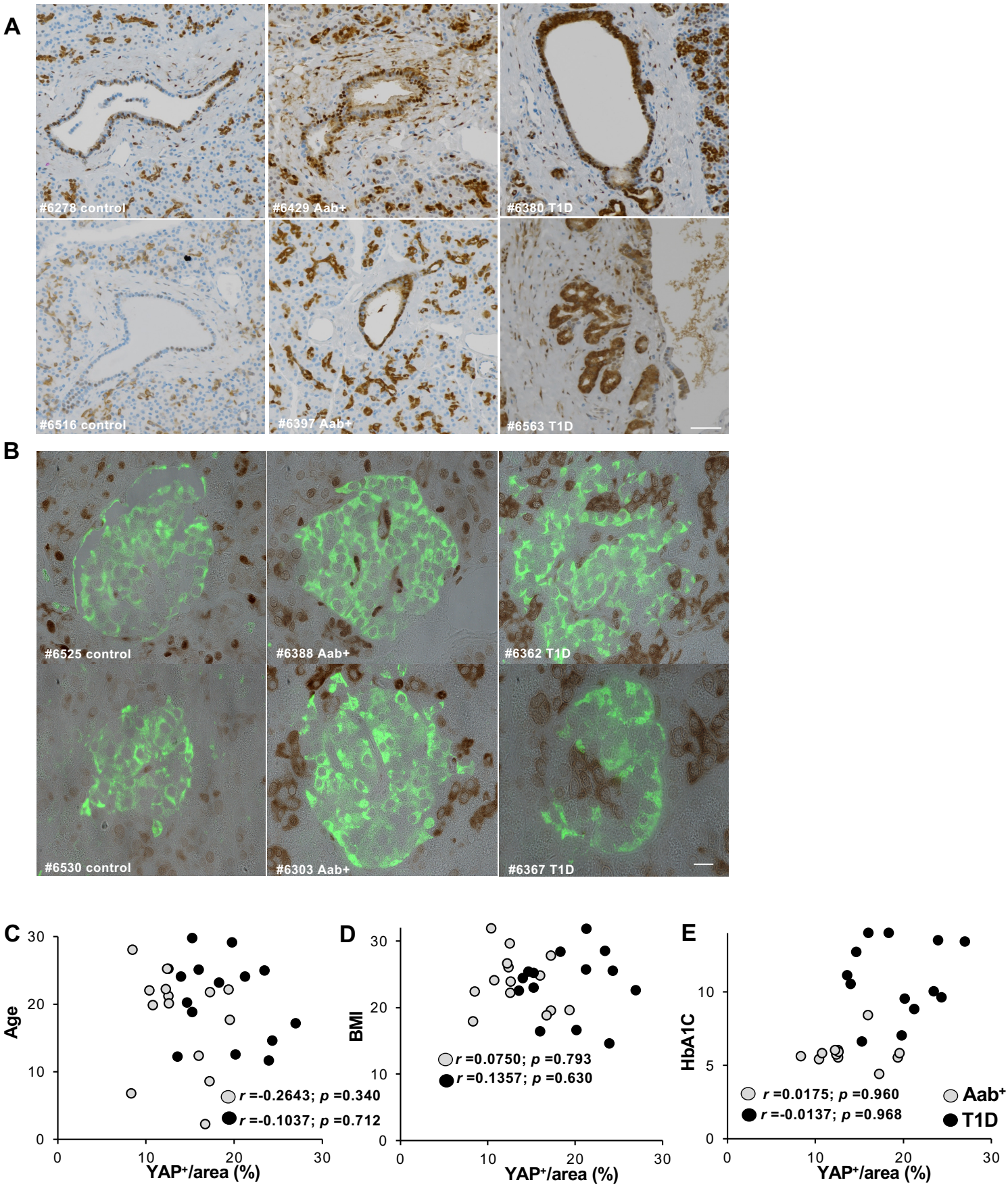

A
#6278 control
#6429 Aab+
#6380 T1D
#6516 control
#6397 Aab+
#6563 T1D
B
#6525 control
#6388 Aab+
#6362 T1D
#6530 control
#6303 Aab+
#6367 T1D
C
Age
YAP+/area (%)
r =-0.2643; p =0.340
r =-0.1037; p =0.712
D
BMI
YAP+/area (%)
r =0.0750; p =0.793
r =0.1357; p =0.630
E
HbA1C
YAP+/area (%)
r =0.0175; p =0.960
r =-0.0137; p =0.968
Aab+
T1D

Figure S1

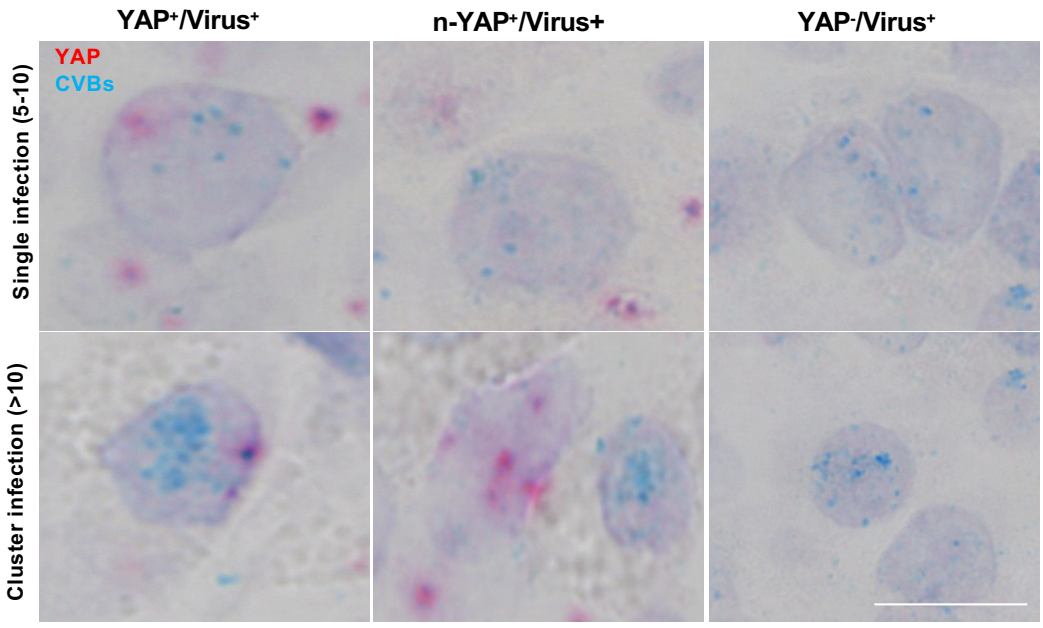

YAP+/Virus+
n-YAP+/Virus+
YAP-/Virus+
YAP
CVBs
Single infection (5-10)
Cluster infection (>10)

Figure S2

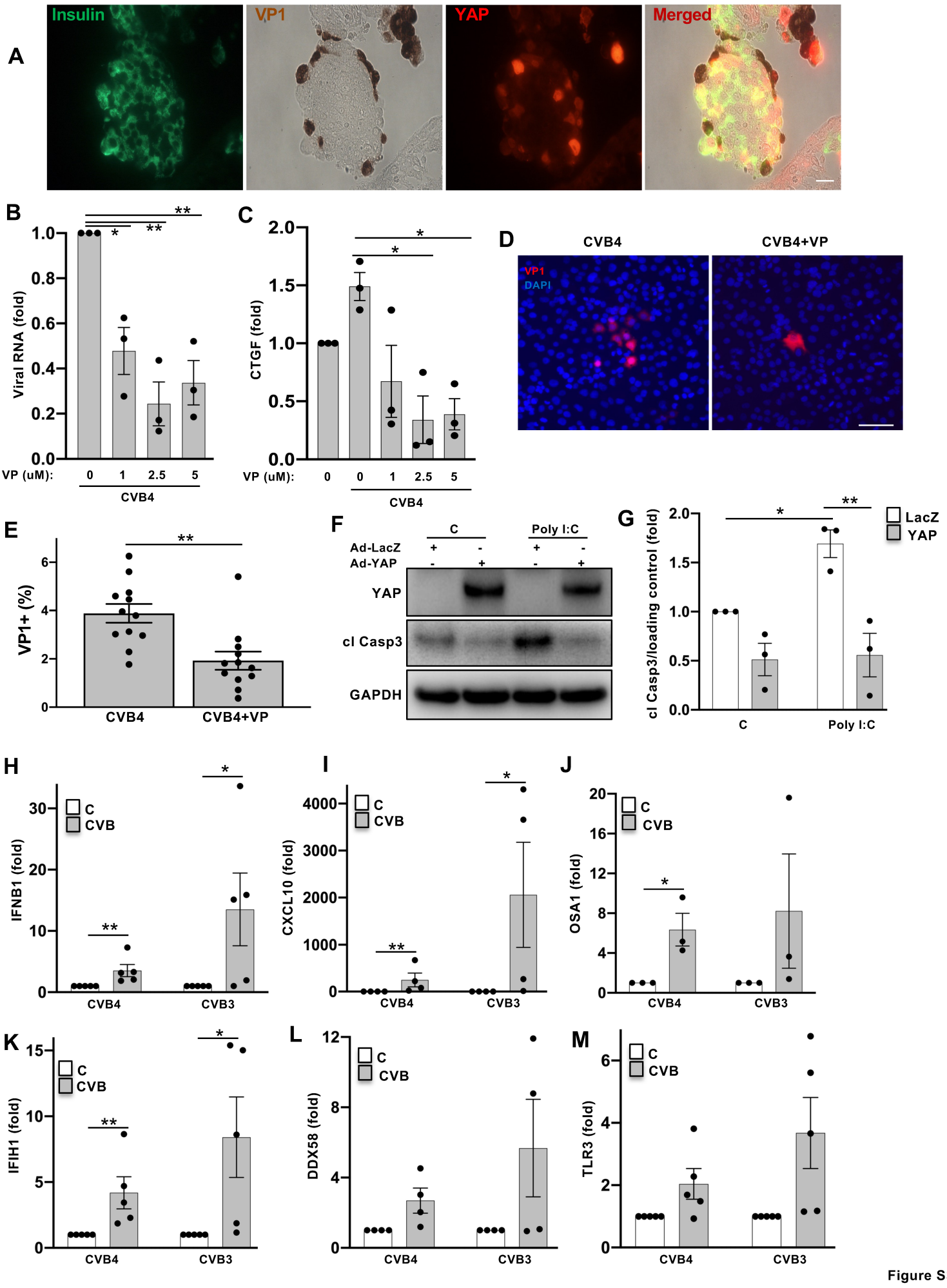

A
Insulin
VP1
YAP
Merged
B
Viral RNA (fold)
VP (uM):
0
1
2.5
5
CVB4
C
CTGF (fold)
VP (uM):
0
0
1
2.5
5
CVB4
D
CVB4
CVB4+VP
VP1
DAPI
E
VP1+ (%)
CVB4
CVB4+VP
F
C
Poly I:C
Ad-LacZ
Ad-YAP
YAP
cl Casp3
GAPDH
G
cl Casp3/loading control (fold)
LacZ
YAP
C
Poly I:C
H
IFNB1 (fold)
C
CVB
CVB4
CVB3
I
CXCL10 (fold)
C
CVB
CVB4
CVB3
J
OSA1 (fold)
C
CVB
CVB4
CVB3
K
IFIH1 (fold)
C
CVB
CVB4
CVB3
L
DDX58 (fold)
C
CVB
CVB4
CVB3
M
TLR3 (fold)
C
CVB
CVB4
CVB3

Figure S3

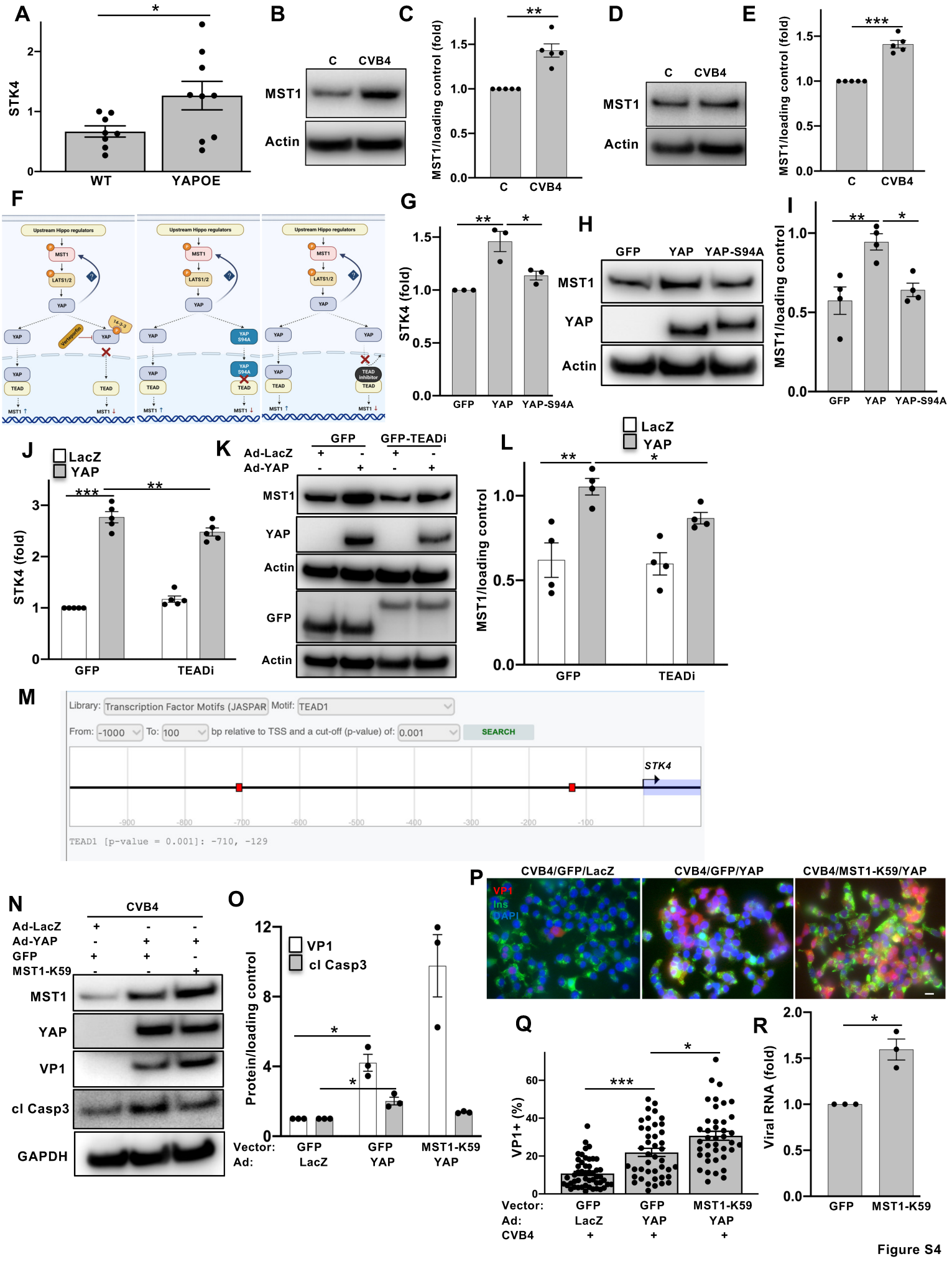

A
STK4
WT
YAPOE
B
C
CVB4
MST1
Actin
C
MST1/loading control (fold)
CVB4
D
C
CVB4
MST1
Actin
E
MST1/loading control (fold)
C
CVB4
F
Upstream Hippo regulators
MST1
LATS1/2
YAP
TEAD
Verteporfin
14-3-3
YAP S94A
TEAD inhibitor
MST1 ↑
MST1 ↓
G
STK4 (fold)
GFP
YAP
YAP-S94A
H
GFP
YAP
YAP-S94A
MST1
YAP
Actin
I
MST1/loading control
GFP
YAP
YAP-S94A
J
LacZ
YAP
STK4 (fold)
GFP
TEADi
K
GFP
GFP-TEADi
Ad-LacZ
Ad-YAP
MST1
YAP
Actin
GFP
Actin
L
LacZ
YAP
MST1/loading control
GFP
TEADi
M
Library: Transcription Factor Motifs (JASPAR
Motif: TEAD1
From: -1000
To: 100
bp relative to TSS and a cut-off (p-value) of: 0.001
SEARCH
STK4
-900
-800
-700
-600
-500
-400
-300
-200
-100
TEAD1 [p-value = 0.001]: -710, -129
N
CVB4
Ad-LacZ
Ad-YAP
GFP
MST1-K59
MST1
YAP
VP1
cl Casp3
GAPDH
O
VP1
cl Casp3
Protein/loading control
Vector:
Ad:
GFP
LacZ
GFP
YAP
MST1-K59
YAP
P
CVB4/GFP/LacZ
CVB4/GFP/YAP
CVB4/MST1-K59/YAP
VP1
Ins
DAPI
Q
VP1+ (%)
Vector:
Ad:
CVB4
GFP
LacZ
+
GFP
YAP
+
MST1-K59
YAP
+
R
Viral RNA (fold)
GFP
MST1-K59

Figure S4

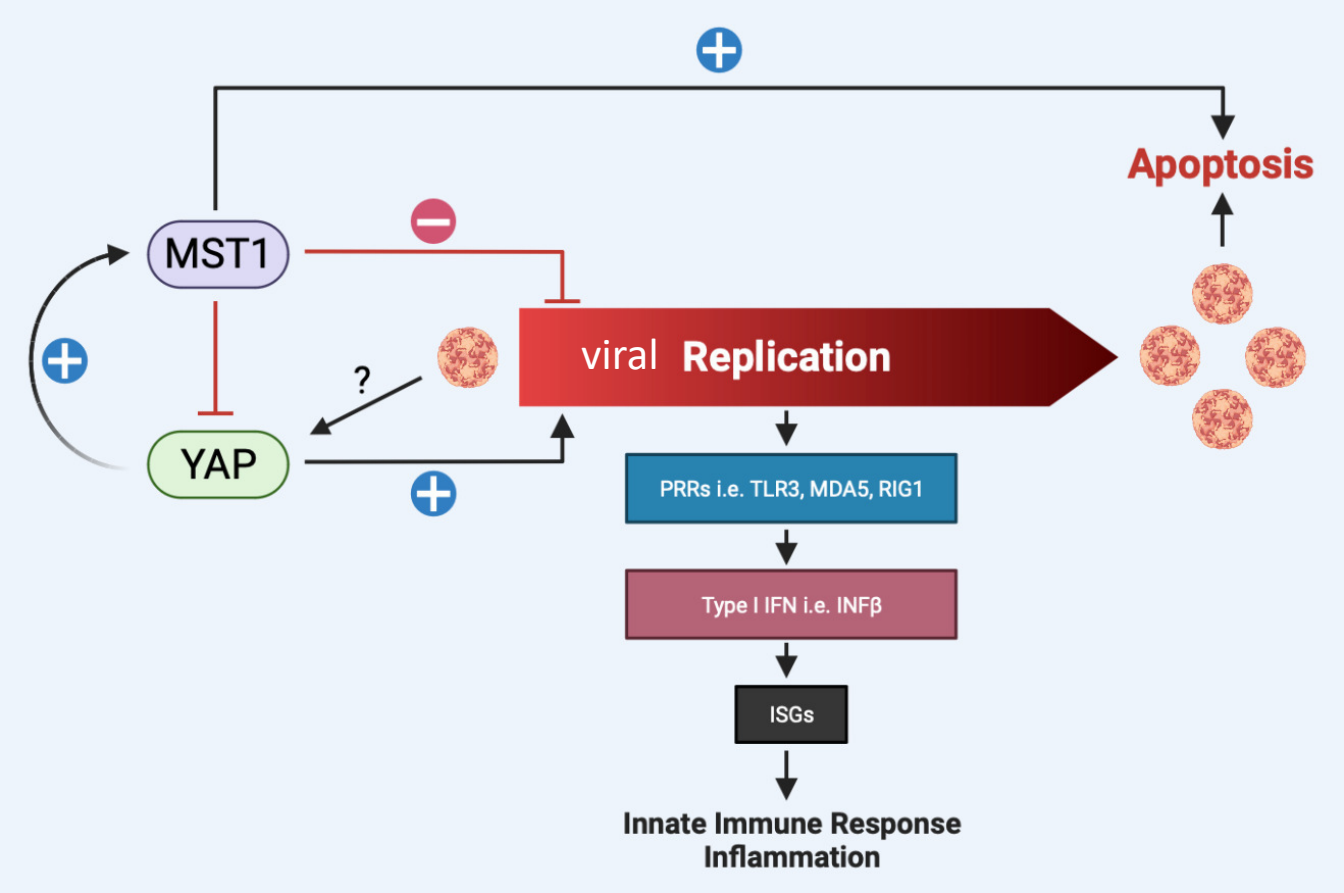

Apoptosis
MST1
viral Replication
?
YAP
PRRs i.e. TLR3, MDA5, RIG1
Type I IFN i.e. INFβ
ISGs
Innate Immune Response
Inflammation

Figure S5

Suppl.Table 1. nPOD inventory donor case IDs and demographics

#	ID	type	Aab	Age	Diab. Durat	gender	Ethnicity	BMI	HbA1c	C-pep (ng/ml)	HLA	Diagnosis
1	6375	control		29	0	male	Caucasian	32	5.7	17.3	A*02/29 B*62/45 DR*04/14 DQ*08/05	Head Trauma
2	6413	control		10	0	female	Caucasian	19	5.6	5.3	A*01/02 B*08/51 DR*01/17 DQ*02/05	Head Trauma
3	6278	control		12	0	female	African American	21	6.3	4.5	A*23/68 B*45/71 DR*11/12 DQ*05/07	Anoxia
4	6384	control		17	0	male	Caucasian	18	4.8	0.7	A*24/69 B*07/55 DR*11/15 DQ*06/07	Head Trauma
5	6254	control		38	0	male	Caucasian	31	5.3	6.4	A*02/29, B*07/62, DR*07/17, DQ*02/02	Anoxia
6	6406	control		7	0	male	Caucasian	17	5.1	4.1	A*02/11 B*44/51 DR*17/07 DQ*02/02	Head Trauma
7	6401	control		25	0	female	Hispanic	31	5.8	12.8	A*31/33, B*14/51, DR*07/13, DQ*02/06	Head Trauma
8	6112	control		6	0	female	Hispanic	18	5.6	5.1	A*26/31, B*51/58, DR*04/13, DQ*05/07	Head Trauma
9	6516	control		21	0	male	Caucasian	29	5.5	8.9	A*26/68, B*44/51, DR*01/07, DQ*02/05	Head Trauma
10	6539	control		25	0	male	Hispanic	19	5.7	39.2	A*02/11, B*27/49, DR*04/13, DQ*06/08	Head Trauma
11	6544	control		12	0	male	African American	23	5.2	8.7	A*32/68, B*18/18, DR*17/11, DQ*02/07	Anoxia
12	6530	control		15	0	female	African American	23	6	7.4	A*03/23, B*45/47, DR*04/17, DQ*02/08	Overdose
13	6525	control		17	0	male	Hispanic	19	5.4	2.7	A*01/02, B*49/51, DR*04/08, DQ*08/04	Anoxia
M				**18**				**23**	**6**	**9.5**		
1	6421	Aab+	GADA+	7	0	male	Hispanic	18	5.6	1.8	A*02/03, B*27/62, DR*01/-, DQ*05/-	Head Trauma
2	6400	Aab+	GADA+	25	0	male	Hispanic	22	5.5	4.2	A*26/31 B*08/62 DR*04/13 DQ*07/08	Head Trauma
3	6429	Aab++	GADA+ mIAA+	22	0	male	African American	20	5.5	2.3	A*01/02, B*44/81, DR*103/17, DQ*02/05	Head Trauma
4	6424	Aab++	GADA+ mIAA+	18	0	male	Caucasian	51	5.8	7.0	A*30/68, B*08/35, DR*17/04, DQ*02/08	Head Trauma
5	6397	Aab+	GADA+	21	0	female	Caucasian	30	6	12.8	A*02/- B*51/62 DR*13/15 DQ*-/-	Head Trauma
6	6303	Aab+	GADA+	22	0	male	Caucasian	32	5.4	3.0	A*01/11 B*44/17 DR*17/07 DQ*02/02	Head Trauma
7	6388	Aab++	GADA+ mIAA+	25	0	female	Hispanic	26	5.7	1.4	A*02/-, B*35/65, DR*01/04, DQ*05/07	Anoxia
8	6310	Aab+	GADA+	28	0	female	Hispanic	22		10.5	A*03/30 B*08/57 DR*07/01	Anoxia
9	6347	Aab+	mIAA+	9	0	male	Caucasian	20		3.3	A*02/32 B*27/61 DR*01/15 DQ*05/06	Head Trauma
10	6090	Aab+	GADA+	2	0	male	Hispanic	19		5.3	A*02/24, B*07/40, DR*04/15, DQ*06/08	Head Trauma
11	6558	Aab+	GADA+			female	African American	28	4.4	8.0	A*02/30, B*08/63, DR*17/07, DQ*02/--	Stroke
12	6532	Aab+	GADA+	20	0	male	Hispanic	24	5.9	22.1	A*02/24, B*13/51, DR*07/11, DQ*02/07	Head Trauma
13	6553	Aab+	mIAA+	12	0	female	Hispanic	25	8.4	4.6	A*02/03, B*62/27, DR*17/04, DQ*02/08	Head Trauma
14	6521	Aab+++	GADA+ IA-2A+ ZnT8A+	20	0	male	Hispanic	24	5.8	7.4	A*02/02, B*08/44, DR*04/17, DQ*02/08	Head Trauma
15	6517	Aab+	GADA+	22	0	male	Caucasian	27	6	9.7	A*03/32, B*38/52, DR*14/15, DQ*05/06	Stroke
M				**18**				**26**	**6**	**6.9**		
1	6299	T1D	mIAA	32	23	male	Caucasian	31.8		<0.05	A*01/11 B*08/55 DR*04/17 DQ*02/08	Anoxia
2	6367	T1D	-	24	2	male	Caucasian	25.7	8.8	0.39	A*02/29 B*18/44 DR*04/07 DQ*02/08	Anoxia
3	6396	T1D	-	17	2	female	Caucasian	22.6	13.4	0.06	A*23/24 B*44/49 DR*07/17 DQ*02/-	DKA, cerebral edema
4	6380	T1D	-	12	0	female	African American	14.6	13.5	0.22	A*33/68 B*71/53 DR*17/13 DQ*02/06	DKA, cerebral edema
5	6371	T1D	GADA+ IA-2A+ mIAA+ ZnT8A+	13	2	female	Caucasian	16.6	9.5	0.11	A*01/68 B*08/65 DR*13/17 DQ*02/06	cerebral edema
6	6211	T1D	GADA+ IA-2A+ ZnT8A+ mIAA+	24	4	female	African American	24.4	10.5	<0.05	A*02/03, B*08/45, DR*04/12, DQ*07/08	Anoxia

7	6414	T1D	GADA+ mIAA+ ZnT8A+	23	0.43	male	African American	28.4	14	0.16	A*01/23 B*07/08 DR*17/09 DQ*02/-	Anoxia
8	6405	T1D	GADA+ IA-2A+ ZnT8A+	29	0.6	female	Hispanic	42.5	7	1.84	A*30/31 B*18/61 DR*04/17 DQ*02/08	Stroke
9	6046	T1D	IA-2A+ ZnT8A+	19	8	female	Caucasian	25.2		<0.05	A*02/03, B*39/62, DR*01/04, DQ*05/08	Anoxia
10	6362	T1D	GADA+	25	0	male	Caucasian	28.5	10	0.38	A*03/11 B*18/35 DR*103/17 DQ*02/05	Head Trauma
11	6563	T1D	IA2A+	15	0	female	Caucasian	25.5	9.6	1.04	A*02/11, B*60/50, DR*07/08, DQ*02/04	Anoxia
12	6536	T1D	GADA+	20	4	female	Caucasian	25.4	12.7	0.04	A*02/31, B*08/60, DR*04/17, DQ*02/08	Anoxia
13	6523	T1D	GADA+ mIAA+	12	3	female	African American	22.5	11.1	0.04	A*24/68, B*08/72, DR*17/07, DQ*02/02	Anoxia
14	6550	T1D	GADA+ ZnT8A+	25	0	male	Caucasian	16.4	14	<0.02	A*33/68, B*08/50, DR*17/--, DQ*02/--	Anoxia
15	6526	T1D	IA-2A+ mIAA+	30	1	male	Hispanic	23	6.6	0.07	A*02/32, B*50/62, DR*04/13, DQ*08/06	Anoxia
M				**21**	**3**			**25**	**11**	**0.3**		

Publication I (Review Article)

Enteroviruses and T1D: Is It the Virus, the Genes or Both which Cause T1D

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Review

Enteroviruses and T1D: Is It the Virus, the Genes or Both Which Cause T1D

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Abstract: Type 1 diabetes (T1D) is a chronic autoimmune disorder that results from the selective destruction of insulin-producing β-cells in the pancreas. Up to now, the mechanisms triggering the initiation and progression of the disease are, in their complexity, not fully understood and imply the disruption of several tolerance networks. Viral infection is one of the environmental factors triggering diabetes, which is initially based on the observation that the disease's incidence follows a periodic pattern within the population. Moreover, the strong correlation of genetic susceptibility is a prerequisite for enteroviral infection associated islet autoimmunity. Epidemiological data and clinical findings indicate enteroviral infections, mainly of the coxsackie B virus family, as potential pathogenic mechanisms to trigger the autoimmune reaction towards β-cells, resulting in the boost of inflammation following β-cell destruction and the onset of T1D. This review discusses previously identified virus-associated genetics and pathways of β-cell destruction. Is it the virus itself which leads to β-cell destruction and T1D progression? Or is it genetic, so that the virus may activate autoimmunity and β-cell destruction only in genetically predisposed individuals?

Keywords: type 1 diabetes; enterovirus; coxsackievirus; beta-cell; HLA; IF1H1; TLR3; IFIH1; YAP; Hippo

1. Introduction

T1D (type 1 diabetes) results from a complex interplay of a multi-genetic predisposition and environmental factors. We have read similar phrases before which are valid for numerous diseases and pathological mechanisms. Saying this is the same for diabetes as well as for any other autoimmune disease: (1) we do not really know what the real cause of the disease is and (2) apparently, there is no single cause for the disease. Thus, this phrase does describe T1D: it results from multiple triggers, which makes the disease very complex. Research has been able to identify many drivers of the disease in the past, such as the initiation of autoimmunity, paths of β-cell destruction, genetic mutations associated with the one (autoimmunity) or the other (β-cell death), or both [1–5].

However, we are still seeking the salient event which finally, through multiple cascades, leads to β-cell failure, loss in insulin production and secretion and, subsequently, hyperglycemia. Protection of the β-cell and prevention of diabetes before its clinical manifestation can be achieved only if the initiators are identified.

What we also know from intensive research is that T1D is a heterogeneous disease. Over the past decades, childhood T1D has increased worldwide at an estimated average annual rate of 3.9%; such doubling during the last 20 years is too high to result only from genetic causes [5–7]. Firstly, the concordance rate between monogenetic twins is only about 50% [8]. Secondly, epidemiological

studies have shown that the disease's incidence follows a periodic pattern within the population [5,6,9] with a significant geographical variation [6].

Support of a putative role for viral infections in the development of T1D comes from epidemiological studies, which have uncovered the seasonal pattern of disease presentation after enterovirus epidemics [6]. Specifically, enteroviruses have been made responsible as an initiator of autoimmunity as well as β-cell failure from epidemiological, pathological and in vitro studies [10–12].

Virus pathology *per se* commonly shows heterogeneity in its outcome, as it causes severe disease only in some affected patients. The current SARS-CoV2 pandemic in 2019/2020 is an overwhelming example of the array of outcomes of virus infection in different people, depending, e.g., on age, genetic background and pre-existing disease, from asymptomatic to pathologic [13]. There is a bidirectional relationship between Covid-19 and diabetes [14]. Firstly, several rapid communications have associated SARS-CoV2 with acute-onset diabetes [14,15], and, secondly, patients with diabetes are at greater risk for severe Covid-19 illness.

Obviously, T1D is not an acute infectious viral disease, as scenarios of massive infection in the pancreas have never been observed in T1D. The virus is lytic to β-cells in vitro, but such has not been detected in vivo, where rather a persistent infection may trigger the immune response. Most of us have had an asymptomatic enteroviral infection during childhood which did not end up causing T1D. With their positive-sense single stranded RNA genome, coxsackieviruses from the family of picornaviridae are widely spread viruses all over the world ranging from 7–22% in Greece and up to 50% and 80% in Montreal and in parts of China, respectively [16]. They most commonly cause hand-foot-and-mouth disease, producing flu-like symptoms, but also have the ability to infect the pancreas, heart and CNS.

Together with an environmental factor, an additional factor is needed to potentiate the susceptibility to enteroviral infections to finally trigger autoimmunity and β-cell destruction, i.e., a certain genetic predisposition. Mutations have been found to either impair virus clearance upon infection, or, oppositely, to increase viral response by inducing a storm of cytokines, which will then destroy the β-cells which are vulnerable to inflammation.

2. Seasonal Patterns of Viral and Autoimmune Diseases

More than 60 infectious diseases have been associated with seasonal patterns, identified by a systematic search for "seasonality" from a list of communicable diseases from the Centers for Disease Control and Prevention (CDC), World Health Organization (WHO), and the European Centre for Disease Prevention and Control [17]. The flu season in the winter of the Northern Hemisphere is the most classic. As enteroviruses and especially coxsackieviruses have multiple serotypes, they cause a broad spectrum of diseases and peak at different times; however, clear seasonality has also been reported for Coxsackie B3 and B4 [17,18].

The seasonal drive is complex and multifarious. There is not only the seasonal viral exposure, but also environmental conditions such as climate (temperature, hours of daylight and sunshine) and human seasonal behavioral, i.e., diet and exercise, which reflects on the host's immune system status and makes us more prone to infection, e.g., to flu in the winter.

In the similar way, most autoimmune diseases "go viral" seasonally, e.g., T1D, multiple sclerosis (MS), systemic lupus erythematosus (SLE), psoriasis, and rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), autoimmune liver diseases (ALDs), autoimmune thyroid disease (AITD), coeliac disease, Sjögren's syndrome (SS) and systemic sclerosis (SSc) [19]. First reported by Franklin Adams in 1926, disease breaks out in the winter season "immediately after such an infection" [9], and this has been later confirmed in large studies [20–22]. T1D diagnosis peaks in the colder months of late autumn to early spring, while it drops in the summer. Such seasonality disappears in regions closer to the equator. Unfortunately, sparse epidemiological data are available from equatorial regions [23], which do not allow any speculation on differences in the T1D incidence *per se*.

In the Finish DIPP cohort study, the appearance of autoantibodies showed a seasonal pattern with a significantly higher proportion in the fall and winter [24]. Thus, autoimmunity follows the

same pattern as viral infection and may not just be directly caused by virus infection, but rather by a combination of unfavorable events at the same time, i.e., higher inflammation in the winter, when diet often changes to sweeter and fattier food with less exercise outside and low vitamin D levels because of limited sunlight exposure, which are all factors that have been independently shown to be associated with T1D [19] (Figure 1). Furthermore, there is the increased risk for another autoimmune disease [25].

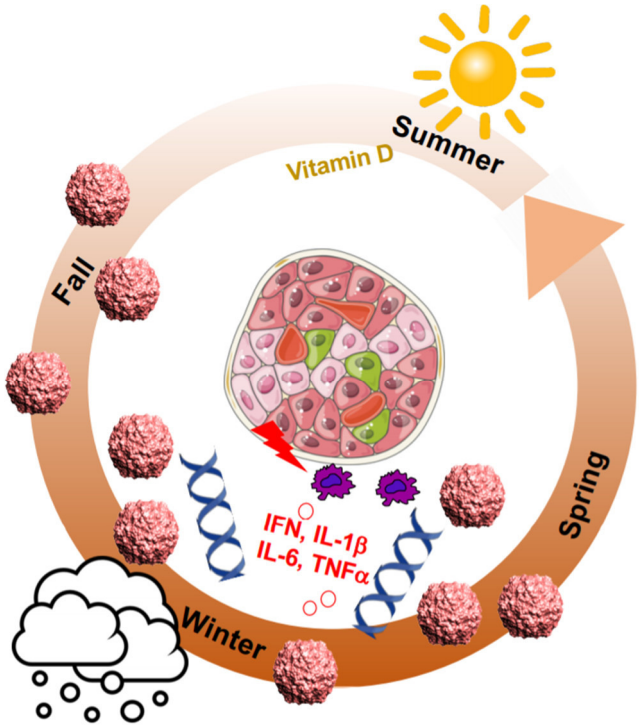

Figure 1. Not only environmental factors but also gene regulation show seasonal patterns. T1D (type 1 diabetes) diagnosis peaks in the colder months of late autumn to early spring, where viral infections come together with less sunlight exposure, less exercise outside, a change in diet together with an increase in pro-inflammatory cytokines and a change towards pro-inflammatory gene networks.

Each of the single factors as a sole initiator for autoimmunity and T1D have been debated and thus, such single factor is unlikely to cause T1D. Early studies from Finland within the DiMe and DIPP cohorts have shown the association of enterovirus infection with autoimmunity and T1D [26–29], while this is not supported by previous results from the DAISY [30] and BABYDIAB [31] cohorts. Another example comes from vitamin D: while several studies show a correlation of lower levels of vitamin D with the onset of T1D [25,32], this was not confirmed by others, and several formulations of vitamin D supplementation could not reduce disease progression [33]. Crucially, it may be the seasonal change in vitamin D metabolism together with changes in the expression of its vitamin D receptor [34] that serve as the additional factors for autoimmune disease predisposition. Using large gene expression datasets from the German BABYDIET, Australia, United Kingdom/Ireland, United States and Iceland cohorts, a previous study also shows seasonal patterns in gene regulation [34]. Gene expression of both the vitamin D receptor and the anti-inflammatory circadian clock regulator transcription factor, BMAL1 (ARNTL1), is lowest in the winter [34], which promotes inflammation through increased levels of soluble IL-6 receptor and C-reactive protein [34]. Several studies in mice and isolated islets show that BMAL1 depletion impairs β-cell survival and disturbs a coordinated insulin secretion which may trigger the onset of diabetes due to defective β-cell function [35,36]. Conversely, BMAL1 is severely depleted in islets from patients with type 2 diabetes (T2D) and disrupted by IL-1β exposure of islets in vitro [37]. This suggests a direct causative role for depleted BMAL1 in inflammation and β-cell failure. Physiologically, the circadian clock would inhibit

inflammation and also prevent the cell from hypoxia, as shown in the heart [38]. Thus, reduction in BMAL1 disables the cellular antioxidant response and increases HIF-1α and ROS accumulation in immune cells, which would further induce the production of proinflammatory cytokines, i.e., TNFα, IL-1β [39] from macrophages, dendritic cells as well as from β-cells themselves [40,41]. The direct cross-talk of transcription factors regulating clock genes (BMAL; ARNTL1) and hypoxia (HIF1α; ARNT) can have fatal consequences. Both belong to same family of PAS-domain, helix-loop-helix transcription factors and share some overlapping DNA binding sites [38,42]. HIF-1α mutations have not only been shown for T1D but also for many other autoimmune diseases [43] and thus again link seasonal changes with genetic predisposition for autoimmune disease. This is especially deleterious for the β-cell with its very low expression of antioxidants and high expression of cytokine and Toll-like receptors [44]. Any increased inflammation may predispose a body to β-cell failure, and thus it may not be the seasonal virus spread alone which causes auto-immunity but rather the pro-inflammatory environment in the host which potentiates β-cell failure with subsequent diabetes initiation. As such, this may only happen in genetically predisposed individuals. All three events together (viruses, the pro-inflammatory milieu in the host and the genetic profile) and their seasonality in their regulation may then initiate β-cell failure and auto-immunity.

3. HLA Class I and Class II Are Major Determiners for T1D

The strongest genetic risk factors for T1D are located in the major histocompatibility complex (MHC, also called the human leukocyte antigen: HLA) class II on chromosome 6, with the predisposing HLA class II haplotypes found in around 90% of patients with T1D [45]; the specific combination of HLA II alleles HLA-DRB1*03 (DR3) or HLA-DRB1*04 (DR4) with DQB1*03:02 (DQ8) confer the highest risk for T1D (for details on HLA susceptibility please see an excellent previous review [46]).

In addition, susceptibility loci also in the HLA I region contribute to T1D [46–48] and their direct association with the age of T1D onset has been shown in several studies [46,47,49]. Predisposing alleles correlate with a younger age, and a protective allele with an older age at onset [47]. Children diagnosed at a very young age usually have a more severe T1D than those diagnosed as teenagers or young adults. Early T1D onset (≤5 years) can predict T1D severity, especially for diabetic complications such as retinopathy [50]. One could assume from these studies that the predisposing HLA class I alleles do not only correlate with age, but also with diabetes severity, although this has not been directly addressed in previous studies. For a possible similar correlation of HLA class II risk alleles with age of onset or severity of disease, only few study results are available. Valdes et al. reported that a DRB1-DQB1 HLA class II at risk allele contributes to the age at onset of T1D. However, a pure prediction of the disease onset from HLA alleles alone has been difficult among populations, since many more factors and their combination, i.e., T1D genetics and auto-antibodies play a major role [47].

The very early appearance of asymptomatic autoimmunity and its strong relationship with age and disease severity was found in all the large prospective T1D studies: BABYDIAB, DIPP (Diabetes Prediction and Prevention) and TEDDY (The Environmental Determinants of Diabetes in the Young). It is detected by any of the ICA, IAA, GAD, IA-2 and ZnT8 auto-antibodies and follows the exponential decay model starting in the first year of life in genetically at-risk children in affected families with first-degree relatives with T1D (FDR). Indeed, children who developed autoimmunity in the first year of life had the highest risk of T1D [51], which is further increased in those children with the high-risk HLA-DR3-DR4-DQ8 or DR4-DQ8/DR4-DQ8 genotypes [24,51].

The strong correlation of HLA-genetic susceptibility as a prerequisite for enteroviral infection-associated islet autoimmunity was depicted many years ago in the Finish DiMe study: children with a high-risk HLA allele converted to ICA positivity during enteroviral infection more often than those without HLA risk [27]. Further results from the DiMe (Childhood Diabetes in Finland) and DIPP studies show increased islet auto-antibody appearance with enterovirus infections during pregnancy and early childhood and their correlation to T1D progression [27–29].

Confirmed in all three major T1D pancreatic tissue biobanks (EADB, Exeter Archival Diabetes Biobank; DiViD, Diabetes Virus Detection Study; and nPOD, Network for Pancreatic Organ Donors with Diabetes), the age of onset determines the number of cases with any left residual beta-cells, i.e., an older age of T1D onset strongly correlates with more remaining β-cells and children with diabetes onset <7 years have fewer β-cells left than at the onset 7–12, and again fewer than those diagnosed at >13 years [52]. Usually seen near disease onset, i.e., within the first 7 years of diagnosis and found located and "hyperexpressed" on the surface of β-cells in T1D [53], HLA I molecules present antigens to activated cytotoxic CD8 T-cells which then lead to islet infiltration and all together to subsequent β-cell destruction (Figure 2). It is therefore possible that such HLA I hyperexpression may coincide with β-cell failure. Although the stimulus for β-cell specific HLA I hyperexpression in vivo is not clear yet, it is often associated with enteroviral infection, indirectly reported based on viral capsid protein immunofluorescence in insulin containing islet (ICI) clusters [52] as well as insulitis. Histological analyses of the human T1D pancreas show all, viral capsid VP1, IFNα, the major cytokine induced by viral infection, and HLA I expressed in or within the islet proximity [52,53]. Mechanistically shown in islets in vitro, enterovirus-induced IFNα [54] leads to β-cell upregulation of HLA class I [55,56]. IFNα-mediated HLA class I induces inflammation and ER stress, but is alone insufficient to cause beta-cell apoptosis. Additional exposure of islets to the pro-inflammatory cytokine IL-1β potentiates β-cell apoptosis [56], suggesting the necessity of a complex pro-inflammatory milieu to induce β-cell failure.

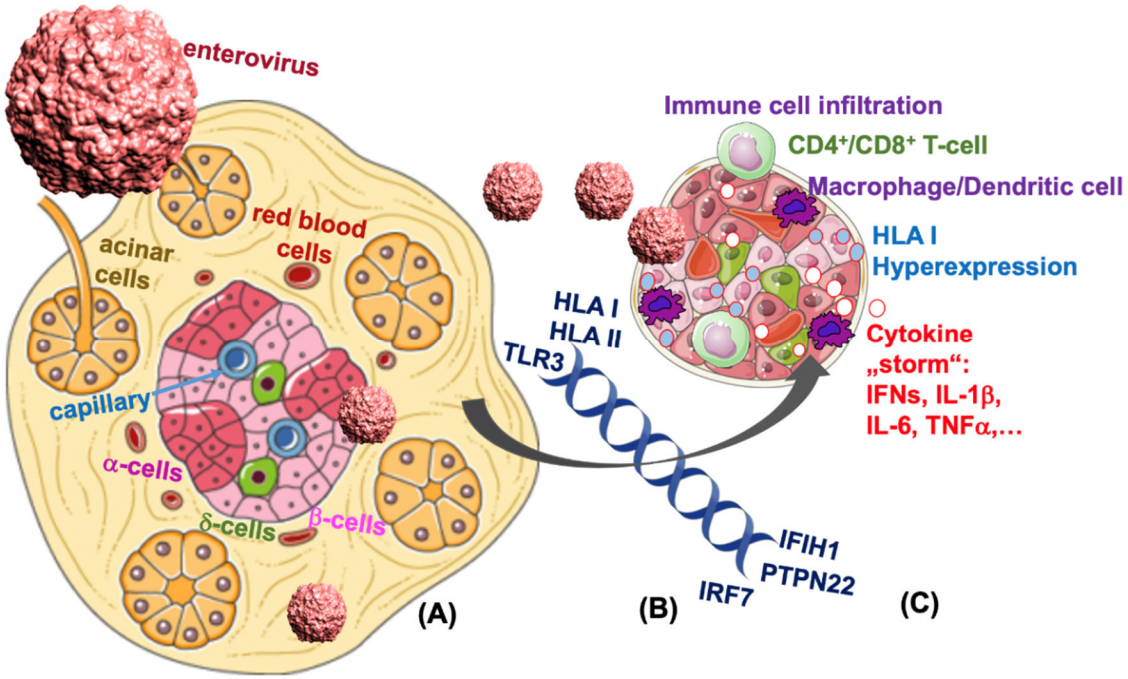

Figure 2. β-cell destruction in T1D is associated with viral response pathways. β-cells are highly vulnerable to enteroviral infection. (**A**) Several genetic mutations in the viral response pathway in T1D may lead to the potentiation in viral response. (**B**) A consequent "storm" of pro-inflammatory cytokines and chemokines lead to HLA I hyperexpression and attract cytotoxic T-cells and macrophages and subsequently to the loss of β-cells (**C**) and manifestation of T1D.

It is important to note that the association of HLA was not only identified for T1D, but for many other autoimmune diseases, i.e., rheumatoid arthritis, celiac disease and multiple sclerosis [19,57] which assumes that (i) physiological HLA is a prerequisite for a balanced immune regulation and (ii) enteroviral infections may lead to disturbance of such balance, through attraction of activated T-cells towards the virus' homing tissue.

Based on these large studies, islet autoimmunity in early life is indeed related to genetic factors and disease severity. The propensity of a very young child, i.e., <1 year to respond to environmental factors such as enteroviruses may thereby potentiate the risk to T1D progression.

4. Direct Evidence for Enteroviral RNA in the Pancreas

Epidemiological data and clinical findings show a correlation between enterovirus infection and the onset of T1D [6,58]. In 1969, Taylor's lab reported the presence of neutralizing anti-coxsackievirus B4 antibodies in the serum of patients with T1D [59]. Since then, enterovirus infections, mainly of the coxsackie B virus (CVB) family, were hypothesized as a potential pathogenic mechanism to trigger the autoimmune reaction to β-cells, resulting in the destruction of β-cells [54,60] and the onset of T1D [61,62]. Following the isolation of CVB4 from a pancreas autopsy of a 10-year-old boy with T1D [63], many large studies tried to identify the virus directly from the T1D pancreas.

In newly diagnosed T1D patients of the DiViD study (3–9 weeks after T1D onset), VP1 was detected in biopsy pancreases in all patients in 1.7% of the islets. It is possible that such a 100% correlation of VP1 and T1D was observed because of a higher expression at diagnosis, which would decline at later stages [64], however such a hypothesis would need to be experimentally proven. Furthermore, HLA I expression was found in all patients. Viral RNA in the frozen pancreas was only found in one T1D patient and from cultured enriched islets in only 4 of 6 patients at a very low concentration (by PCR, >40 cycles), which shows no evidence of an acute but, if any, rather a low-grade infection. In confirmation with several previous studies [65], classical RT-PCR was not sensitive enough for the analysis of a viral infection, which only occurs in few cells within the whole pancreas. RNA sequencing from the whole pancreas could not identify any viral sequences, again suggesting the threshold of the presence of viral sequences compared to all other genes as sparse to be identified by classical RNASeq methods. Nevertheless, several approaches have confirmed the presence of enteroviruses both in the circulation and in islets of T1D patients [64,66–70], however, because of a very low expression, many attempts have failed to characterize the localization and the specific enteroviral sequences through PCR-based methods in the pancreas.

Enrichment strategies are necessary to detect such low-grade infection, e.g., amplification of viruses by preculturing human leucocytes from patients with T1D and subsequent RT-PCR analysis [71] or by the elegant viral-capture sequencing methods in which viral sequences are enriched before sequencing, that enable the identification of enteroviruses in stool samples from islet auto-antibody positive children [72].

Viruses that have a specific tropism within the islets could cause the onset of the disease not only by direct cytolysis but also by triggering the host immune response [73]. The presence of several CVB viruses, including CVB4, together with the Coxsackie-adenovirus receptor (CAR) in the β-cell, support the connection of viral infection with T1D. Coxsackieviruses induce a persistent, slowly-replicating infection; this may result from alterations to the viral genome during the progress of infection, such as naturally occurring 5′-deletions [74–76]. Because of several such limitations to the detection of enteroviruses, we have previously established an adapted method to target single RNA molecules with short (~20 nucleotides) fluorescently labeled oligonucleotides in situ. Probes consist of a mixture of 40 short oligonucleotides covering the whole length of the viral genome and anneal to common regions of the RNA genome of the coxsackievirus family [77]. This enables targeting single RNA molecules. Short labeled oligo RNA probes are more resistant to RNAse, and RNA detection is less affected by target RNA degradation and fragmentation. Through the availability of the well-characterized cohort of human pancreatic donor tissue established by nPOD [78], viral mRNA can be detected in the T1D pancreas with high sensitivity, specificity and accuracy and at lower viral loads than by classical immunostaining and even PCR [77,79]. Further ongoing studies of pancreas sections revealed remarkable significance of viral RNA expression in T1D pancreata, compared to controls without T1D [80].

Using this method, we have analyzed whole pancreas sections and quantified enteroviral mRNA by unbiased scans and identified viral mRNA distributed not specifically within or in proximity to islets; enteroviral mRNA was evident through individual dots in single cells throughout the pancreas (Figure 3). Such observation is in contrast to VP1 immunohistochemistry in the pancreas [81], which mostly detected VP1 positivity in or near islets. Famously referred to as the "streetlight effect" [82], it is difficult to find what we search for in the dark, and thus, it is possible that several antibody-based stainings were preferentially observed in islets, although the staining has been carefully re-evaluated

and VP1 correlates with hyperexpression of HLA class I in islets [83]. The commonly used DAKO-VP1-Ab detects several other antigens in addition to VP1 and/or exocrine enzymes may degrade enteroviral proteins and thus prevent their detection in the pancreas [84].

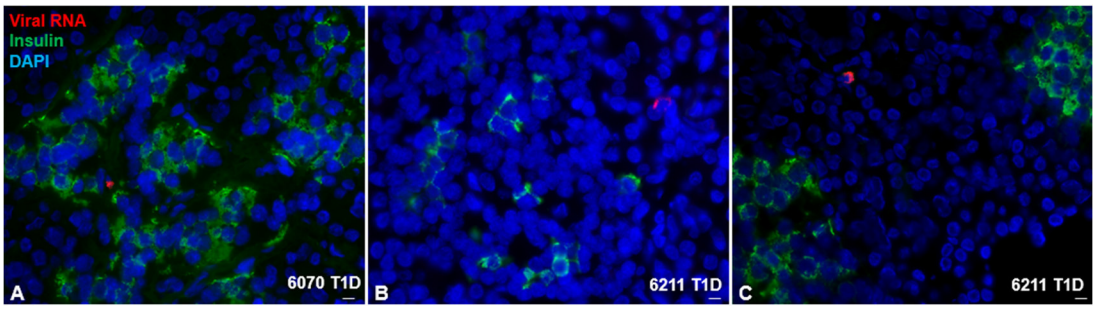

Figure 3. Coxsackieviral RNA in the T1D pancreas**.** Representative images of T1D donors 6070 and 6211 from the nPOD cohort. Viral RNA was found within the endocrine area (**A**) and outside the islets (**B**,**C**) shown by co-staining of viral RNA probes (red), insulin (green) and DAPI (nuclei; blue). Tissues were first probed for viral RNA, and then stained for insulin after a previously established protocol ([77]; Busse et al.). Scale bar depicts 10 μm.

Early studies, where C57BL/6 mice were infected with CVB3, also observed viral infection localization in the pancreas in the acinar cells, together with severe inflammation and acinar cell destruction [85]. Despite the well-known differences in enteroviruses' tropism in the pancreas in mice and humans [85], such observation is in line with the decreased acinar cell number and acinar tissue mass reported in numerous studies from human T1D pancreases [86–88].

Rather than from the virus itself, β-cell destruction may result from "bystander" damage [89,90], where coxsackie virus infection may lead to a storm of inflammation in cells like the β-cell, which carry an enormous amount of pattern recognition (such as TLR3 and TLR4), cytokine (such as IL-1R1), and chemokine receptors on their surface [44]. Their activation by viruses and by cellular viral responses stop viral replication on one hand, but induce tissue damage on the other. In addition, interferons accelerate expression of surface HLA-I molecules and thus activation of auto-reactive T-cells against β-cells (Figures 2,4). T-cell activation through non-T-cell receptors ("bystander damage") [89] is limited to viral infection [91], where β-cell apoptosis is triggered by viral response products, e.g., cytokines and chemokines [92]. Such a pro-inflammatory environment has also been shown to alter the composition of the islet extracellular matrix, which may further facilitate T-cell migration towards pancreatic islets [93]. The specific and severe β-cell destruction then occurs through their special vulnerability towards an array of cytokines and chemokines such as interleukin (IL)-1β, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and CXCL10 [94], which induce β-cell destruction in response to viral infection in human islets [60,95] (Figure 4). It is also possible that multiple infections during childhood each time contribute to potentiating the immune response and then lead to β-cell destruction, autoimmunity and T1D.

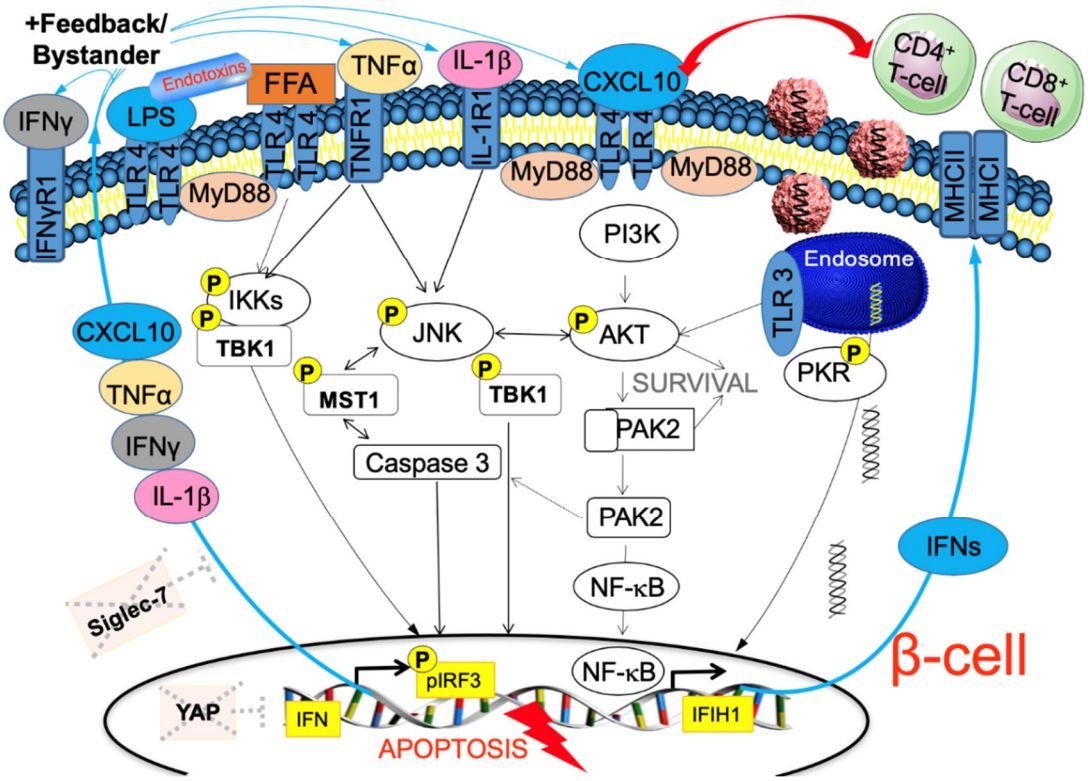

Figure 4. β-cell in the storm. Our hypothetical model on how chronic potentiation of proinflammatory pathways leads to β-cell destruction. Coxsackieviruses enter the β-cell through the Coxsackie–adenovirus receptor (CAR) and bind to endosomal TLR3. While the virus promotes the AKT-JNK axis for initial host cell survival, parallel activation of viral response pathways through PKR-TBK-IRF3 leads to the transcriptional activation of the IFN response and production of interferons, which increase surface MHCs, recognized by cytotoxic CD8- and CD4-T-cells causing "bystander damage", and β-cell apoptosis through a "storm" of cytokines and chemokines, which all find their receptors on the surface of the β-cell, and a vicious cycle is initiated with the full activation of the apoptotic machinery including JNK-MST1-Caspase 3-NFκB. Bacterial toxins as well as chronically elevated free fatty acids (FFA) are also associated with β-cell damage and act through TLR4 activation and similar downstream pro-inflammatory pathways. While many cells can counteract such damage cycles with a potent survival machinery, the β-cell is deficient of the Hippo terminator YAP, which would balance the viral IRF3 response. Furthermore, Siglec-7, which balances immune activation, is diminished in a chronic diabetogenic pro-inflammatory milieu in the β-cell.

5. Enteroviral Infection and T1D: Results from the TEDDY Study

Recent results from the large multi-center TEDDY cohort study provided important confirmation of the association of enteroviral infection and islet autoimmunity [96]. Direct next-generation sequencing of stool samples as well as analyses subsequent to cell culture amplification of enteroviruses identified an array of DNA and RNA viruses.

The study confirmed that enterovirus B infections (EVB) were associated with islet autoimmunity, but also examined the role of length of infection since sequential stools from children were available. An association with islet autoimmunity was detected with long-duration enterovirus B infections, indicated by prolonged shedding of the same virus in multiple stool samples. In contrast, multiple independent short-term enterovirus B infections without prolonged shedding neither correlated with autoimmunity nor with T1D progression.

The results of this study indeed reproduce a correlation of enteroviral infection and autoimmunity: that the duration of the virus load detectable in stool samples determines the progression to autoimmunity. Mechanistically, one can assume from this and many previous studies that the virus may trigger autoimmunity, but is not conclusively linked to further T1D progression.

This is in line with data showing that the enteroviral signaling cascade, which leads to the IFN response, is increased before auto-antibody conversion and T1D (see above) [97], which again suggests virus infection and the boosted IFN response as primary event toward autoimmunity. It is very likely that the longer duration of enterovirus abidance in the host is defined by the genes and their unfavorable seasonal changes.

Once a host is found, the virus creates a variety of smart mechanisms to escape from anti-viral immune response through persistent infection, e.g., blocking autophagy in order to remain in the cell [16]. Dysfunctional autophagy as a feature of both T1D and T2D [98] supports such hypothesis. Enteroviral B's typical 5′ terminal genomic deletions observed in cardiomyocytes [99] and in the pancreas [76] may lead to a long term stay of viruses in the cell without causing lysis. This probably enables detection of viral RNA in autopsy pancreata even a long time after occurrence of islet auto-antibodies [80] as well as after T1D diagnosis [77] in morphologically normal appearing cells.

Highly sensitive virus-captured sequencing methods from stool samples also confirm the association of enteroviral infection with islet autoimmunity [72], and enteroviral amplification-enrichment cultures of leukocytes and of cells from duodenal biopsies showed the correlation of enteroviruses B and T1D. A significant association between enterovirus and subsequent risk of autoimmunity in celiac disease was also found in TEDDY and other previous studies [57,100], where enteroviral positive stool samples correlated with celiac disease only after introduction of gluten to the babies' diet [57], and higher amounts of gluten consumption potentiated the effect of enteroviruses on the risk of coeliac disease autoimmunity [100], indicating the necessity of the initial autoimmunity trigger.

6. TLR3 Signaling Leads to Enterovirus-Induced β-Cell Destruction

The innate immune response to virus infection initiates as a fingerprint with the sensing of viral pathogen-associated molecular patterns (PAMP). Such recognition is mediated by the activation of host's pattern recognition receptors (PRR) such as Toll-like receptors (TLR) on the surface of cellular membranes and cytosolic receptors including RIG-like receptors (RLR), nucleotide-binding domain-leucine-rich repeat-containing molecules (NLR) and RNA-activated protein kinase R (PKR) [101]. Many studies show that the onset of diabetes is triggered through PRRs [102–104], and PRRs have been identified as susceptibility factors for diabetes progression in genetic studies [105–107]. Most of the today's described 10 human TLRs, namely TLR2-4 and 6-9 have been associated with T1D or/and T2D [108,109]. There is a strong correlation of the most TLR3 polymorphisms with T1D in several [107,110] but not in all studies [111].

TLRs are used by the immune system for pathogen clearance. The endosomal receptor TLR3, found not only in immune but also various non-immune cells such as the β-cell, is one of the signaling complexes implicated in viral-mediated β-cell death, is highly expressed in the pancreas of patients with T1D [112] and is found enhanced in human islets by IFN exposure [113]. Once viral RNA is recognized by TLR3, the TLR3-TANK binding kinase 1 (TBK1)-IFN-regulatory factor (IRF)3/7 signaling axis is activated [114]; the virus initially induces AKT [60] to make sure that its host survives but later cross-talks with JNK result in activation and translocation of NF-κB subunits to the nucleus (Figure 4).

Downstream of the viral response pathway is the C-X-C motif chemokine 10 (CXCL10) which promotes human β-cell apoptosis [94]. CXCL10 is localized in infected islets [115] in both canonical and fulminant T1D early in disease progression [44,94] and thus is suggested as a clinical marker for diabetes onset [116]. The cascade finally ends in the secretion of proinflammatory chemokines and cytokines, which further potentiate inflammation and β-cell apoptosis pathways (Figure 4).

Several studies in mice have shown that TLR3 is an essential element of T1D development in response to viral infection. As a detector of viral signatures, TLR3 is needed for the anti-viral response, and, naturally, will promote cytokine signaling. These two apparent conflicting effects towards beta-cell survival may provide reasons for various different results in mice and imply that a highly balanced physiological function of viral sensors is necessary to prevent damage to β-cells. TLR3 signals contribute to the host's survival, as CVB4 [117] or encephalomyocarditis virus [118]

infections are highly mortal to TLR3 knockout mice due to the impaired antiviral response machinery. Although they present a reduced pro-inflammatory milieu, surviving mice develop T1D [117]. Other studies show that TLR3 knockout in NOD mice has no effect on the incidence of diabetes at a basal level [119] and that CVB4-infected TLR3 knockout NOD mice show lower diabetes incidence [120]. In the absence of TRIF, a prominent downstream protein in the TLR3 cascade, mice are also protected from the development of T1D by changing the gut microbiota [121].

In summary, pattern recognition receptors identify viral antigens to trigger the host defense. TLR3 signaling through multiple loops leads to virus-mediated inflammatory response, and ongoing inflammation further potentiates the cytokine response through multiple cytokine and chemokine receptors expressed in the β-cell, and finally to β-cell apoptosis in vitro. However, as many examples show, mutations in a single PRR, e.g., TLR3, or its activation alone will not ultimately cause T1D, but may rather act within a pro-inflammatory network to potentiate T1D progression (Figure 4). A future research target towards prevention could therefore be specific miRNAs, as many of them which are differentially expressed in T1D patients [122] are involved in the regulation of the innate as well as the adaptive immunity through TLR signaling [123].

7. IFN-Inducible Genes Link Autoimmunity, Viral Response and β-Cell Failure in T1D

T1D is associated with over 60 genetic risk regions across the human genome, identified by genome-wide association studies (GWAS) [124], and these T1D-linked SNPs alter the expression of over 200 genes [125] involved in β-cell inflammation, function and destruction, immune activation and signaling, including viral response, Toll-like receptor, cytokines and NF-*κ*B signaling. Among them, several risks as well as protective single nucleotide polymorphisms within the interferon-induced helicase-1 (*IFIH1*) gene, which encodes the melanoma differentiation associated protein 5 (MDA5), have been identified in large studies [106,126]. IFIH1 is a cytosolic sensor of single strand viral RNA from the picornavirus family. It facilitates the interferon (IFN) response and activates the immune cells towards viral response downstream of TLR signaling. Importantly, expression of the IFN signature genes as well as the type 1 IFN response is increased in children before the T1D-associated auto-antibody conversion [97,127], which suggests a primary role of IFN signals in the activation of autoimmunity and the potentiation of β-cell destruction. In β-cells, IFN signaling leads to HLA class I hyperexpression, which is a well-studied path for T1D initiation [106,128]. *IFIH1* is ultimately associated with signals from enteroviruses; its mRNA expression is increased by CVB3 and CVB4 infection in human islets [114] and by synthetic double-stranded RNA Poly(I:C) in INS-1E β-cells [129], while *IFIH1* silencing potently lowers the chemokine response in β-cells [129]. Foremost, a diabetes-associated *IFIH1* polymorphism upregulates the IFN signature in human pancreatic islets in response to Coxsackievirus infection [130].

The upregulation of IFN-inducible genes, including *IFIH1* in genetically predisposed children, was also associated with previous upper respiratory tract infections and with increased monocytic expression of the sialic-acid binding immunoglobulin-like-lectin Siglec-1 [97]. Through the recognition of specific glycans on the cell surface, Siglecs promote cellular interactions within the immune system and with sialylated pathogens; they are important regulators of the innate and adaptive immune systems and serve as checkpoints for immune regulation and autoimmunity [131]. Through their immunoreceptor tyrosine-based inhibitory motifs (ITIMs), Siglecs balance the immune response [132]. Several members of the Siglec family do not only play a role in immune–cell–pathogen interactions, but also on the level of the β-cells regulate the inflammatory response. Siglec-7 is down-regulated in both β-cells in the pancreas from patients with T1D and T2D as well as in activated immune cells. Overexpression of Siglec-7 in diabetic islets balances the immune response by reducing cytokine production and monocyte migration, which both facilitate β-cell survival and function [133]. The evolving field of Siglecs provides a further target to modulate the excess inflammatory/IFN response as a major facilitator for autoimmunity and β-cell failure.

8. Why the Beta-Cell? Absence of the HIPPO Effector YAP to Balance Viral Response

Despite certain viral tropisms, viral receptors are distributed in many cells in all organs and IFN-induced viral defense mechanisms are in place, which (i) hinder viral reproduction and (ii) attract cytotoxic T-cells. In the largely non-replicative β-cells, such an increase in the IFN response seems deleterious. The intracellular antiviral defence is initiated by TBK1-IRF3-mediated interferon production (see Sections 6 and 7 above) [134] and controlled by the Hippo terminators and transcriptional regulators YAP and TAZ [135–137], which negatively regulate and thus balance the antiviral immune response. Recent studies have linked YAP/TAZ with antiviral sensing [135–137]. YAP/TAZ associate with both TBK1 and the inhibitor of nuclear factor kappa-B kinase (IKKε), thereby blocking their activation and subsequently inhibiting IRF3-stimulated transcription of viral response genes. Thus, YAP/TAZ, besides their well-known function in the regulation of cellular contact, development, growth and proliferation as effectors of the Hippo pathway [138], can regulate the host's cellular response. In the absence of this YAP regulation, virus sensing would trigger an extremely high and uncoordinated cytokine response, as happens in T1D, where virus-infected β-cells show highly increased cytokine production resulting in a vicious cycle and bystander damage of β-cells through their cytokine receptors (Figure 4).

One underlying reason could be the absence of YAP in adult β-cells. During endocrine cell differentiation, YAP is suppressed as soon as Ngn3 is expressed [139,140]. The lack of YAP expression correlates with the extremely low rate of β-cell proliferation and β-cell quiescence after birth and their limited regenerative capability [141]. The Hippo element YAP is sufficient to wake β-cells up from quiescence; re-expression of constitutively active YAP leads to a robust induction of human β-cell proliferation [140,142]. Similarly, TAZ is extremely low but detectable in both adult human and mouse β- and α-cells [143,144]. Bioinformatic analysis identified YAP as a selectively repressed ("disallowed") gene in the pancreatic islet [145]; it is more repressed in purified mouse β-cells compared to α-cells [146]. Now, we hypothesize this as the reason not only for the much lower proliferative capacity of β-cells compared to any other endocrine cell type, but also for the extreme and suicidal viral response. In contrast, the Hippo kinase MST1 represses antiviral signaling and acts as negative regulator of the antiviral defense by its direct interaction and phosphorylation of IRF3 and inhibition of TBK1 [137]; however, underlying mechanisms as well as consequences on host survival are not known. Previous data from our and other labs show that Hippo is an important regulator of β-cell function and survival [139,140,147], and therefore it may also be involved in the pathological viral response associated with T1D.

9. Conclusions

There is large evidence for enteroviral infection initiating the auto-immune response and subsequent β-cell destruction in genetically predisposed individuals, where a viral response is boosted. As an especially vulnerable cell to inflammatory destruction and apoptosis, autoimmunity is directed to the β-cell, causing T1D. Although enteroviruses selectively and severely destroy β-cells in vitro, they are just one stimulating factor in the huge complexity of T1D, and thus, without an unphysiological genetic predisposition towards immune activation and β-cells' inability for compensation, they would probably not cause T1D. Therefore, it is possible that enteroviral vaccination and antiviral therapies for T1D [148], although they would take away the stimulus, may alone not be sufficient to cure the disease and require combination with further β-cell protection efficacy. This is reminiscent of gluco- and lipotoxicity-mediated β-cell failure associated with T2D [149]. Although highly toxic for the β-cell in vitro, elevated glucose and free fatty acids only induce some alterations and systemic compensation as long-term consequences of obesity in vivo. However, in genetically predisposed individuals, they finally lead to T2D [150]. Similarly, neither viral infections alone nor predisposing genetic polymorphisms alone ultimately lead to T1D. As there is no single cause for T1D, we will probably not be able to successfully cure diabetes with a single drug. Rather, forces need to join for testing the efficacy of combination therapies, for example antiviral strategies [148] together with the prevention of T-cell action [151], anti-inflammation [152] and/or beta-cell protection [153].

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Conflicts of Interest: Page: 12
The authors declare no conflict of interest.

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4 Discussion

The damage and dysfunction of insulin-producing β-cells is the underlying causes of T1D and T2D. Enterovirus infection, especially by CVB is thought to be a major trigger of T1D. The Hippo pathway is a prominent regulator of organ size and tissue homeostasis and critical for the regulation of β-cell function and viability. We identified that the key effector of the Hippo pathway, YAP, enhanced coxsackievirus replication and potentiated coxsackievirus-induced islet inflammation and β-cell apoptosis. Conversely, inhibiting YAP expression reduced coxsackievirus replication in the pancreas but improved β-cell survival.

Coxsackievirus B4 was first isolated from the pancreas of a child with diabetic ketoacidosis, together with islet lymphocytic infiltration and β-cell necrosis [1]. The in depth analysis of the pancreas tissue by smFISH enabled to detect significantly increased enterovirus RNA in the pancreas of both T1D and AAb+ donors [2].

Are enteroviral infections unique to T1D or is it also related to T2D?

To investigate whether enterovirus infection is associated with T2D, enterovirus smFISH RNA expression analysis was performed in pancreas tissue of T2D donors. Viral RNA+ cells were rarely seen in islets, and similar pattern was shown between controls and T2D donors, except an only particular case with large amount of virus expression. Quantification showed no correlation between enteroviral infections and the onset of T2D.

In contrast, Richardson *et al.* observed strong VP1 signals in the islets from T2D patients, and hypothesized that enteroviruses may not only specific to T1D but also to T2D [3]. However, immunostaining is less sensitive and less specific, compare with smFISH. A non-specific immune response with the VP1 antiserum and the suspicion of the source of the previously isolated virus may also affect the VP1 signal [4], [5]. Even when they analyzed for the presence of enteroviruses using two other antisera against enterovirus proteins, they still could not eliminate the nonspecific reactivity of antibodies and cross-reactivity against other antigens. The more important difference in our study was that we counted the number of viruses in the whole pancreas and distinguished whether they were located in exocrine cells or endocrine cells. In contrast, the two studies before only assessed the infection rate by calculating the ratio of the number of cases with strongly positive cells within islet to the total number of cases [6].

Although enteroviral infection has indeed been detected in pancreatic tissue from patients with both T1D and T2D, we still cannot exclude that there is an association between enterovirus infection and T2D pathogenesis. What they all have in common is islet inflammation [7], [8]. Both types of diabetes are characterized by progressive β-cell failure, leading to absolute or relative insulin deficiency. Studies have shown intra-islet expression of inflammatory mediators, triggering a final common pathway of β-cell apoptosis, progressive β-cell loss, and both T1D and T2D [9], [10]. In our study, we observed a significant reduction in insulin area and an increase in lymphocytic infiltration in the pancreas of individuals with T2D, which confirmed this.

In T1D (Figure 1), viral infections as environmental triggers can active autoimmune responses which mediate progressive β-cell death [11], [12]. When a susceptible individual is exposed to certain viruses, the virus may trigger an immune response that not only targets the virus but also activates autoreactive T cells [13], [14]. These autoreactive T cells then start attacking the β-cells, leading to their destruction and subsequent insulin deficiency [15], [16]. In T2D, the primary problem is insulin resistance and metabolic dysfunction. Viral infections, particularly those that cause systemic inflammation, can lead to increased insulin resistance by promoting inflammation and interfering with insulin signaling pathways [17], [18]. In addition, viral infection, such as influenza A, cytomegalovirus and herpes simplex were also shown that reduce systemic insulin sensitivity [19], [20]. This combination of reduced insulin sensitivity and increased insulin resistance exacerbates hyperglycemia and the development of T2D in susceptible individuals.

In T1D, viral infection can direct cytolysis of β-cells or cause local inflammation [4], [21]. Viral induced pro-inflammatory cytokines production, such as IL-1β, IL-6, IFN-γ and TNF-α, can induce β-cell apoptosis and contribute to the development of insulitis [22], [23]. Insulitis also induces upregulation of MHC class I and II on β-cells to further increase β-cell immunogenicity [24]. In T2D (Figure 1), Chronic low-grade inflammation is now recognized as an important factor contributing to insulin resistance and T2D [25]. In obese individuals, adipocytes release pro-inflammatory molecules called adipokines, which induce adipose tissue inflammation. These adipokines, such as TNF-alpha and IL-6 can promote inflammation and interfere with insulin signaling, contributing to insulin resistance [26]–[28]. TNF-α leads to increased production of fatty acids by adipocytes, resulting in increased releases of free fatty acids, which can weaken insulin signaling [29]. Inhibiting insulin signal transduction may also decrease insulin secretion [30], [31]. In our study, we observed that many CD45+ lymphocytes were located in the exocrine zone and close to virus-infected cells, and the infiltration of the lymphocyte marker CD45 was significantly increased in the islets of T2D patients. Consistently, Steenblock *et al.* demonstrated that islets

show immune cell infiltration with a general increase in inter-islet CD45-positive cells in patients with COVID19 [32]. SARS-CoV-2, similarly to CVBs can directly attack β-cells and induce β-cell impairment and death [33].

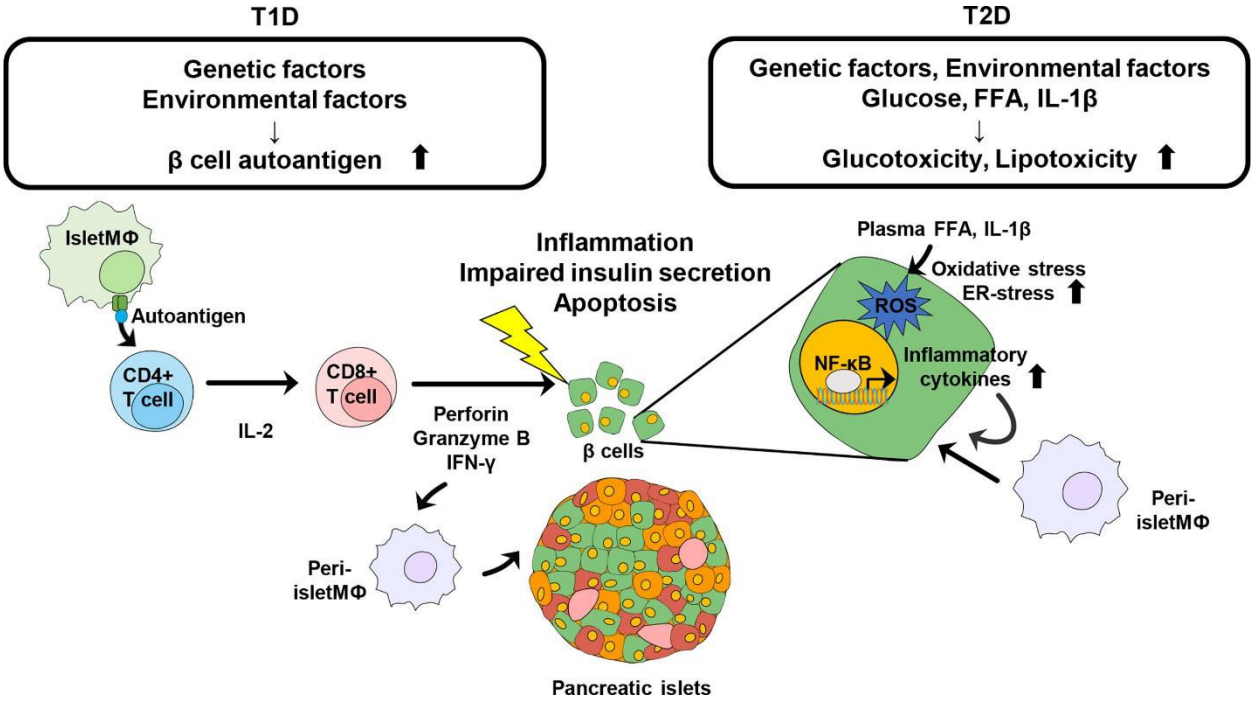

Figure 1. Inflammatory signals in T1D: Activation of immune cells is involved in pancreatic beta-cell death through a variety of inflammatory cytokines. Environmental factors contribute to generation of islet autoantigens. APCs and T lymphocytes participate in the inflammatory processes that promote the development of T1D. **T2D:** Obesity, glucotoxicity and lipotoxicity are contribute to the development of T2D. FFA, and IL-1β promote oxidative stress and ER-stress in pancreatic β-cells, activation of NF-kB pathway induce inflammatory cytokines production, promote insulin resistance as well as β cell destruction.

Importantly, in T1D, the release of inflammatory cytokines and the inflammatory response are unique to islet inflammation. In T2D, the inflammatory response is systemic. We can observe the inflammatory response not only in the pancreas, but also in other organs of the body.

Hippo-YAP, possible to regulate viral activities in viral-induced T1D? Beneficial or unbeneficial?

Despite substantial evidence supporting an association between enterovirus infection and T1D, the complex interactions between enterovirus-host are still poorly understood. Previous studies from our laboratory have shown that modulation of the Hippo pathway promotes pancreatic β-cell proliferation and improves their function [34]. Recent studies have shown that the Hippo pathway is involved in viral activity [35]. A variety of viruses, such as hepatitis B virus (HBV) [36]–[38], hepatitis C virus (HCV) [39], [40], human papillomavirus (HPV) [41]–[43], molluscum contagiosum virus (MCV) [44], Epstein-Barr virus (EBV) [45], [46], Zika virus (ZIKV) [47], etc., can manipulate Hippo pathway to create an environment favorable for their survival, and further affect viral replication, cell proliferation and transformation. However, a large number of studies have shown that YAP, as a double-edged sword, can not only enhance the host's antiviral immune response to ensure cell survival [48], [49], but also reduce the host's antiviral ability and promote inflammation [50]–[53].

Thus, we wanted to know whether YAP promotes or reduces enteroviral replication in virus-induced type 1 diabetes. First, we examined the endogenous expression of YAP in the exocrine and endocrine pancreas by performing YAP immunohistochemical (IHC) and Yap mRNA expression analyses by using high sensitive RNAscope technique on paraffin-embedded pancreatic tissue from organ donors from nPOD (Network for Pancreatic Organ Donors with Diabetes). We observed hyper-expressed YAP in exocrine and endocrine pancreas of AAb+ and T1D organ donors compared to non-diabetic controls. It is no coincidence that YAP is upregulated in AAb+ and T1D, as it may suggest that YAP may stop the development of T1D progress by promoting cell survival during increased viral activity. Alternatively, the virus can recruit YAP to create a favorable environment for its own replication and proliferation, that's why we could find more YAP around virally infected cells.

LATS1/2 kinases, the major upstream effectors that phosphorylate and inactive YAP/TAZ, by regulating YAP/TAZ can effectively reduce the inhibition of TBK1/IKKε. This shows a way to balance YAP/TAZ-associated antiviral responses [48]. Conversely, YAP deficiency results in enhanced innate immunity suggesting that YAP can also negatively regulate antiviral innate immune responses [49]. To investigate the potential link between YAP expression and CVB replication, our work showed that YAP enhanced coxsackievirus replication and potentiated coxsackievirus-induced islet inflammation and β-cell apoptosis. We observed a significant

potentiation in β-cell apoptosis triggered by coxsackievirus infection and pro-inflammatory cytokines in adenovirus-YAP treated INS-1E β-cells and isolated primary human islets.

The TEAD transcription factor family mainly binds to YAP and mediates YAP-induced gene expression [54]. A better understanding of the interaction of TEAD with YAP will provide insights into the immunomodulatory role of the Hippo pathway in various diseases [55]. In addition to uncovering the initial intracellular molecular mechanisms that interfere with viral replication, pharmacological modulation of the Hippo pathway provide strategy to understand the mechanisms of antiviral responses. For example, verteporfin (VP), an FDA-approved drug and inhibitor of the YAP/TAZ-TEAD complex, has anti-cancer effects in different types of cancer [56]–[58]. In our study, VP showed a clearly reduced CVB3 and CVB4 replication, which indeed supports the Hippo pathway and TEAD as its target as virus potentiator. Also in the human ductal cell line PANC1, VP inhibition of YAP significantly abrogated CVB4 RNA genome replication. Several other paths can interact with TEAD, for example Vestigial-like family member 4 (VGLL4) which has the similar function as VP to eliminate the interaction between YAP and TEAD [59]. Also statins, known as HMG-CoA reductase inhibitors, can inhibit YAP-induced transcription by retain YAP and TAZ in the cytoplasm [60]. Both should be investigated in the future for a possible T1D-abrogating effect.

In T1D, the immune system attack on the β-cells primarily affects the pancreas endocrine function by disrupting insulin production. However, exocrine abnormalities are an important but mostly overlooked feature associated with T1D [61]. Our results demonstrate that there is substantial colocalization of CVB infection and YAP in pancreatic exocrine cells. As the exocrine part of the pancreas represents 96 to 99% of its volume [62], [63], we have reason to suspect that the failure of pancreatic endocrine cells may be caused by pancreatic exocrine infection, and the potential reason may be that YAP migrates from exocrine to endocrine and induces virus replication. In support of tinfection starting in the exocrine pancreas, massive neutrophil infiltration in the exocrine pancreases were seen in two patients with T1D who died at disease onset [64]. Also, YAP is commonly upregulated in T1D as well as PDAC and pancreatitis, suggesting that the Hippo/YAP pathway may play a pervasive and central role in the pathogenesis of pancreatic disease [65], [66]. In our cell culture model of human exocrine pancreatic cells, YAP overexpression directly promoted CVB replication and enhanced β-cell apoptosis and expression of genes involved in innate immunity and antiviral defense.

MST1 is a critical effector of Hippo pathway and acts as a pro-apoptotic molecule, leading to β-cell failure under diabetic conditions [67]. Interfering with MST1 expression or activity can reduce

apoptosis and limit YAP-mediated uncontrolled cell proliferation and cell expansion [68]–[70]. In our experiments, adenoviral YAP overexpression lead to upregulation of MST1. This indicates that finally, YAP may balance antiviral responses through an upregulation of MST1, consisting of a positive feedback regulatory pathway that interconnects YAP with MST1.

Firstly, we found a YAP-MST1 negative feedback loop; inhibiting MST1 resulted in increased YAP activity, subsequently leading to higher viral replication but decreased β-cell apoptosis. A similar phenomenon was also described by SARS-CoV-2 infection. YAP promotes viral replication and production of SARS-CoV-2, while MST1 inhibits SARS-CoV-2 replication [71].

To confirm the existence of this loop between MST1 and YAP, we further performed experiments to identify YAP-TEAD-induced transcription of STK4. Our results shown that VP blocked YAP induced MST1/STK4 expression. Secondly, overexpression of the YAP-S94A, which has a TEAD-binding domain-deficient mutation [72], failed to induce MST1 upregulation. This suggests that YAP-induced MST1 activity can only occur with TEAD. Thirdly, a small-molecule TEAD inhibitor (TEADi) that blocks the interaction between YAP and TEAD [73], [74] also downregulated MST1/STK4. All these show that the transcription of STK4 is primarily mediated by YAP-TEAD interaction, which increases the abundance of MST1 protein and constitutes initially a positive feedback loop from YAP to MST1. Thereafter, MST1 completely blocks YAP through classical Hippo signalling, and thus the final loop is negative.

We believe that our newly discovered YAP-MST1 feedback loop is essential in the regulation of viral replication and β-cell death during enterovirus infection, providing potential therapeutics targeting β-cell healing and protection.

We emphasize its importance during viral infection because of the synergistic effect of YAP/MST1 in viral infection, which results in immunosuppression and virus-induced apoptosis [67], [71]. This inhibition allows the virus to replicate freely, which then increases apoptosis in pancreatic β-cell [33], [35], [67].

In this work, we revealed an essential role of YAP as key host factor for enteroviral amplification in the pancreas. Modulating YAP may be beneficial for virus-mediated β-cell injury in T1D. It is necessary to emphasize the interplay between viral infection and the Hippo pathway as an area of important future research. Future studies focusing on the precise role of YAP in response to viral infection will provide insights into pathogenesis, and contribute to the development of potential therapeutic strategies targeting YAP against virus-induced diabetes or β-cell failure.

Summarizing Remarks

1. Our findings suggest no link between enteroviral infection of the pancreas in patients with T2D compared to age and BMI-matched non-diabetic controls.
2. There is a significant increase in the general lymphocyte marker CD45 in the islets of patients with T2D, where the insulin area is significantly decreased, compared to non-diabetic controls.
3. YAP is hyper-expressed in the pancreas of T1D donors and donors with Aab, which positively correlates with increase of enteroviral infection.
4. YAP potentiates CVB3 and CVB4 replication in β-cells and acts as pro-inflammatory factor, while YAP inhibition limits viral amplification.
5. There is a negative feedback loop mechanism between YAP and the core kinase of the Hippo pathway, MST1, where YAP upregulation increases the expression of MST1 to finally limit the actions of YAP and viral replication.

Future Perspectives:

The results of my study highlight the role of virus infection in diabetes. I introduce the involvement of the Hippo signaling pathway in viral-induced T1D. The following are some of the questions that could be addressed in subsequent studies:

1. Is there any particular virus that could be involved in the development of T2D?
2. What is the significance of the exocrine-endocrine crosstalk?
3. How can higher YAP expression in the exocrine cells lead to more viral infections in β-cells and T1D?
4. How does viral infection change the expression of YAP?
5. How might expression of LATS1/2 or other Hippo players change due to YAP overexpression? What role would they play in viral amplification?
6. Could YAP inhibition be a logical approach for preserving β-cells from infection and apoptosis?

Subsequent studies could significantly contribute to our understanding of the complex interplay between viral infections, cellular signaling pathways, and the development of T1D.

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Appendices

Contributions in Research Articles

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

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

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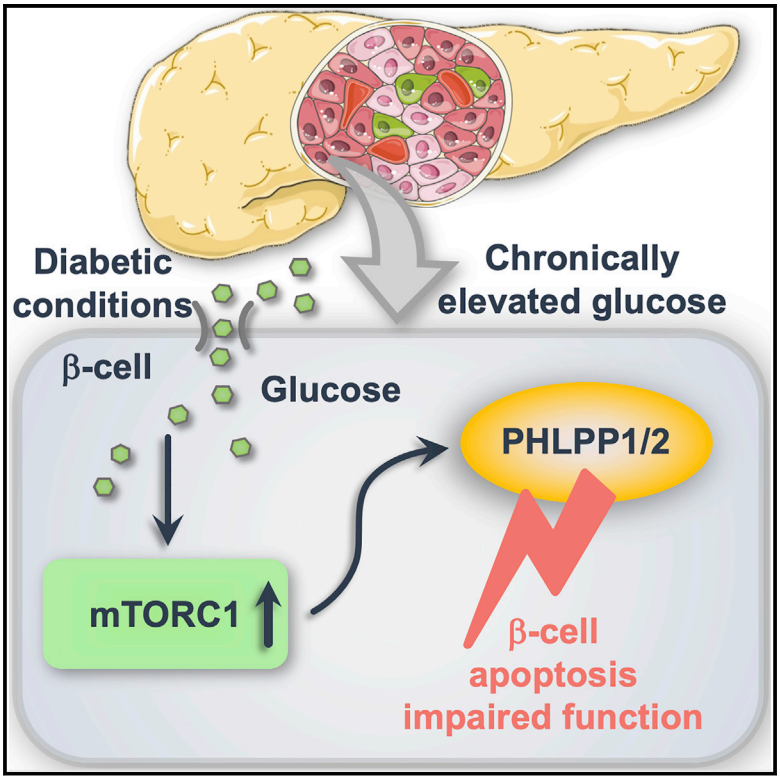
My contribution:
Performed mouse islet isolations and western blot experiments in Figures 2,3,5 (partially).

Cell Reports

Article

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

Graphical abstract



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In brief

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

Highlights

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

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Article

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

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SUMMARY

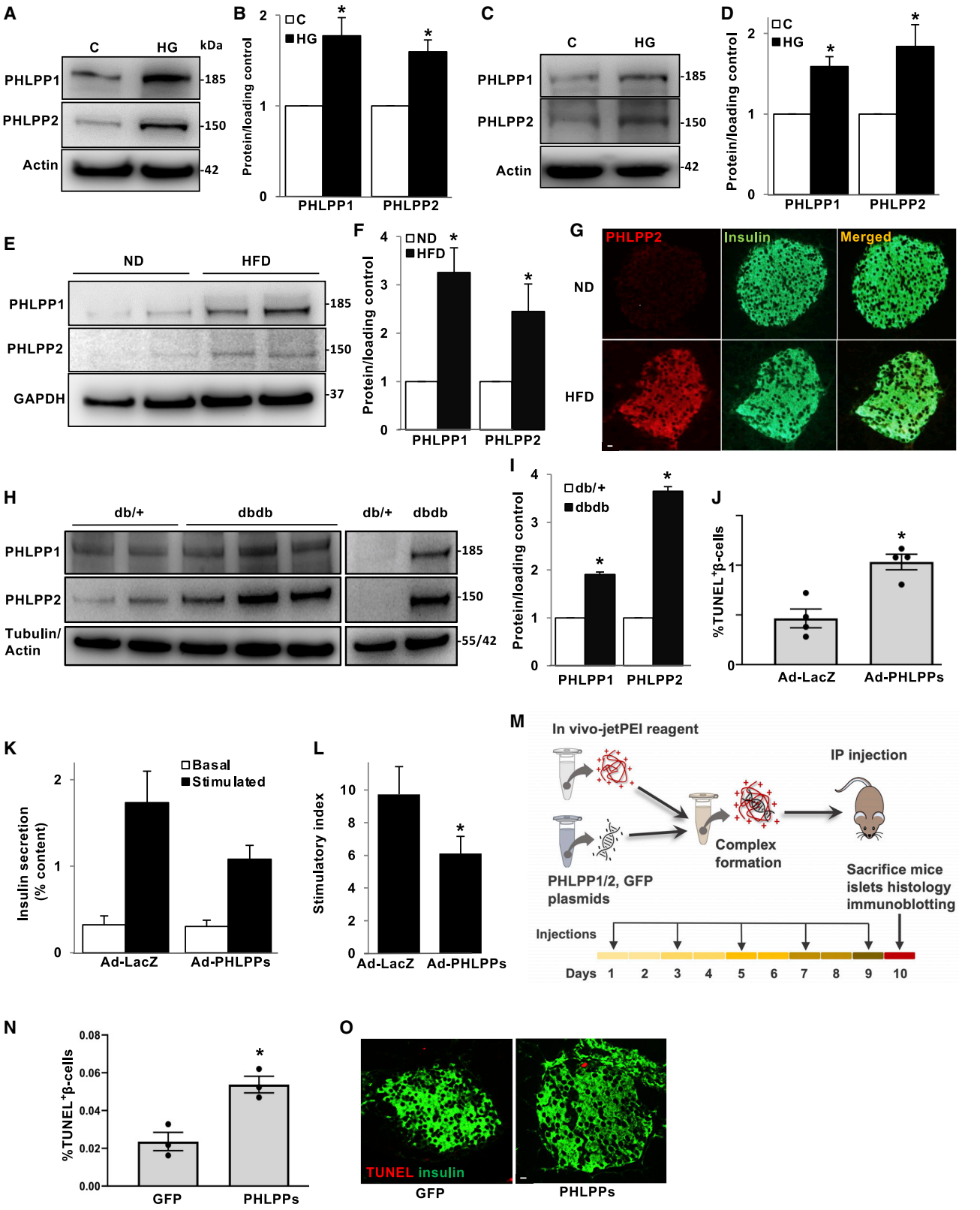
Pancreatic β-cell failure is the key pathogenic element of the complex metabolic deterioration in type 2 diabetes (T2D); its underlying pathomechanism is still elusive. Here, we identify pleckstrin homology domain leucine-rich repeat protein phosphatases 1 and 2 (PHLPP1/2) as phosphatases whose upregulation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

INTRODUCTION

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

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

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



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

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

RESULTS

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

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

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

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

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

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

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

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

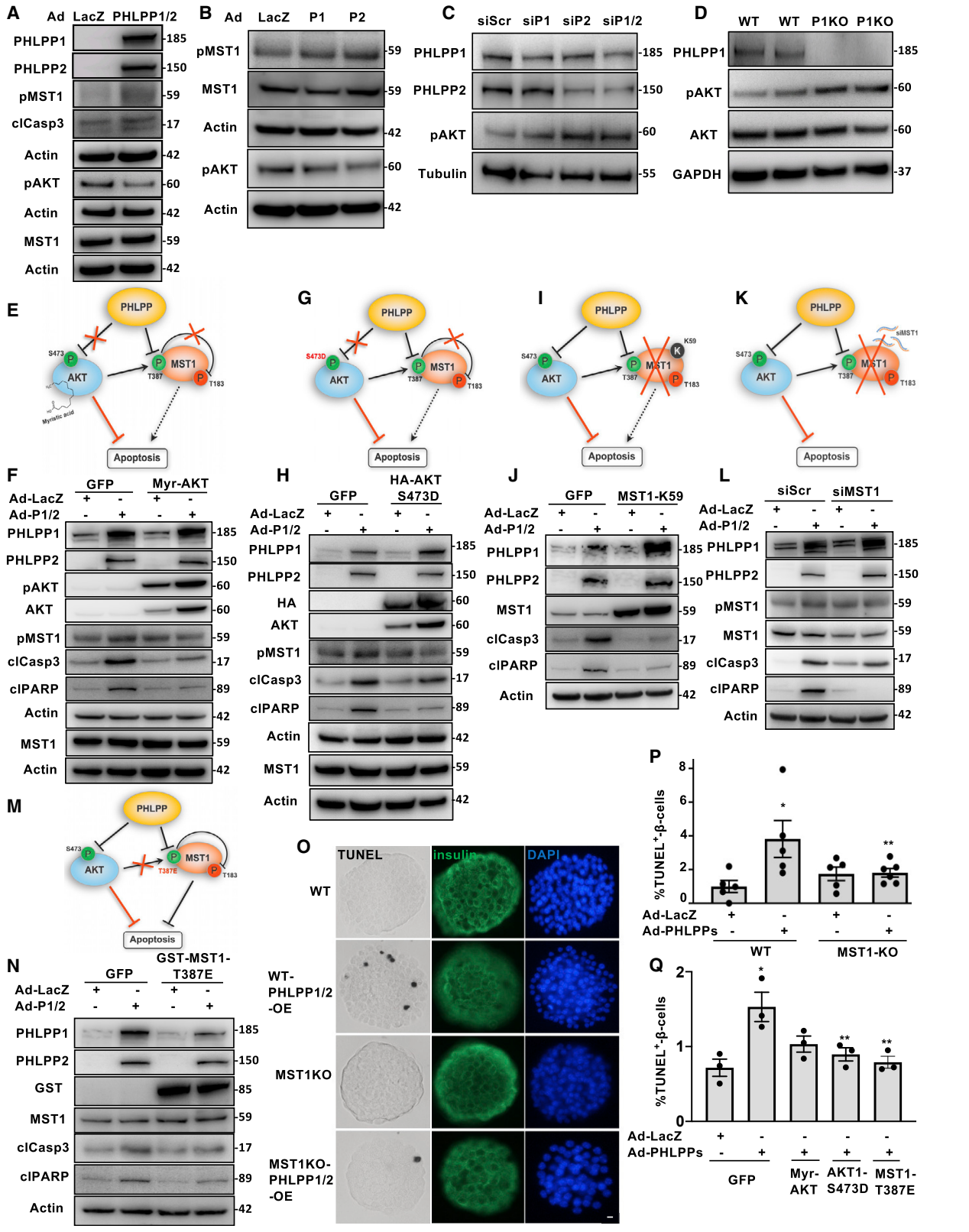
(H and I) Representative western blots (H) and quantitative densitometry analysis (I) of isolated islets from 10-week-old diabetic *db/db* mice and their heterozygous *db/+* littermates (n = 5).

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

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

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

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



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

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

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

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

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

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

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

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

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

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

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

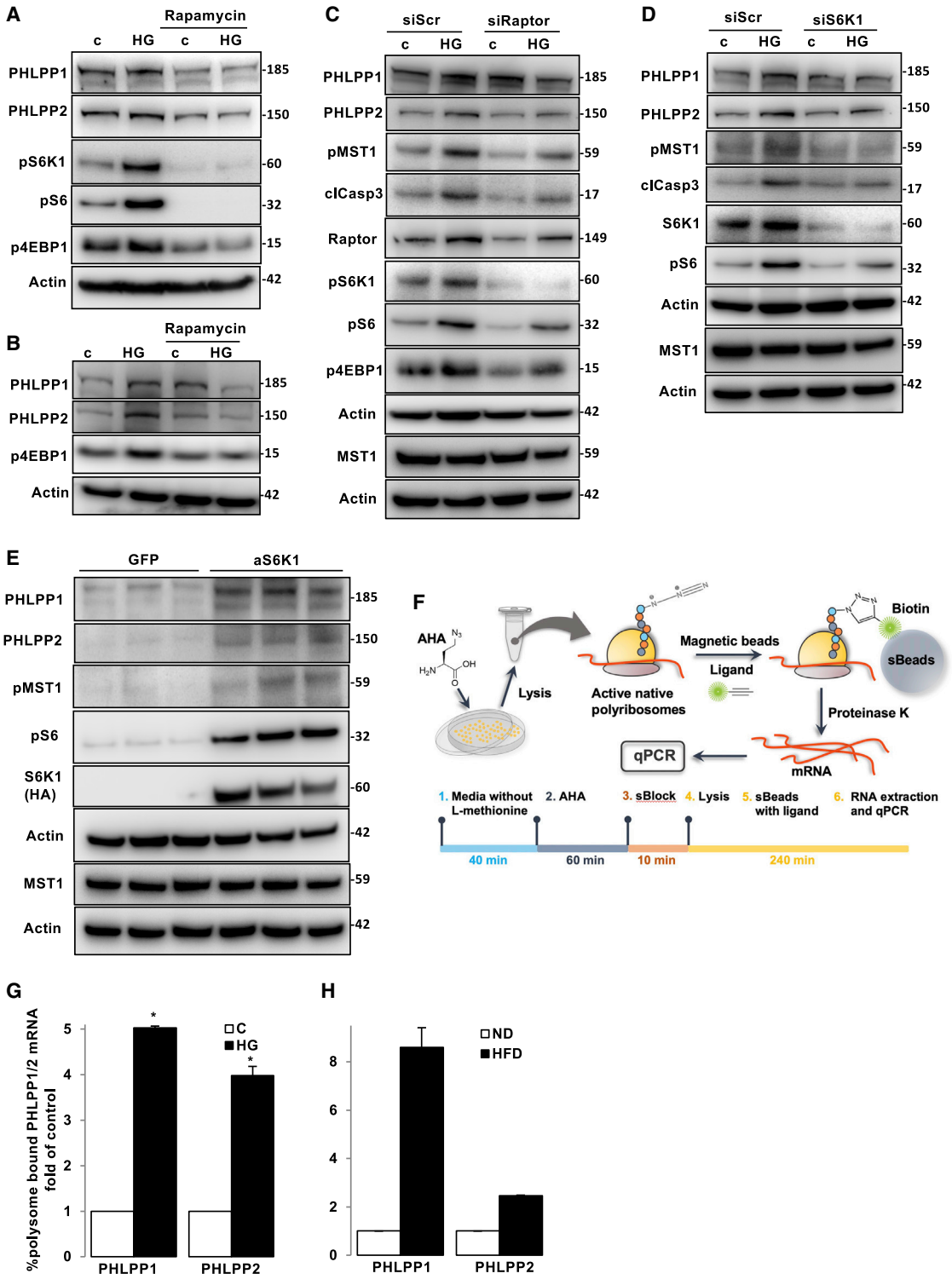

Figure 3. mTORC1 hyper-activation induces PHLPPs translation

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

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

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

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

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

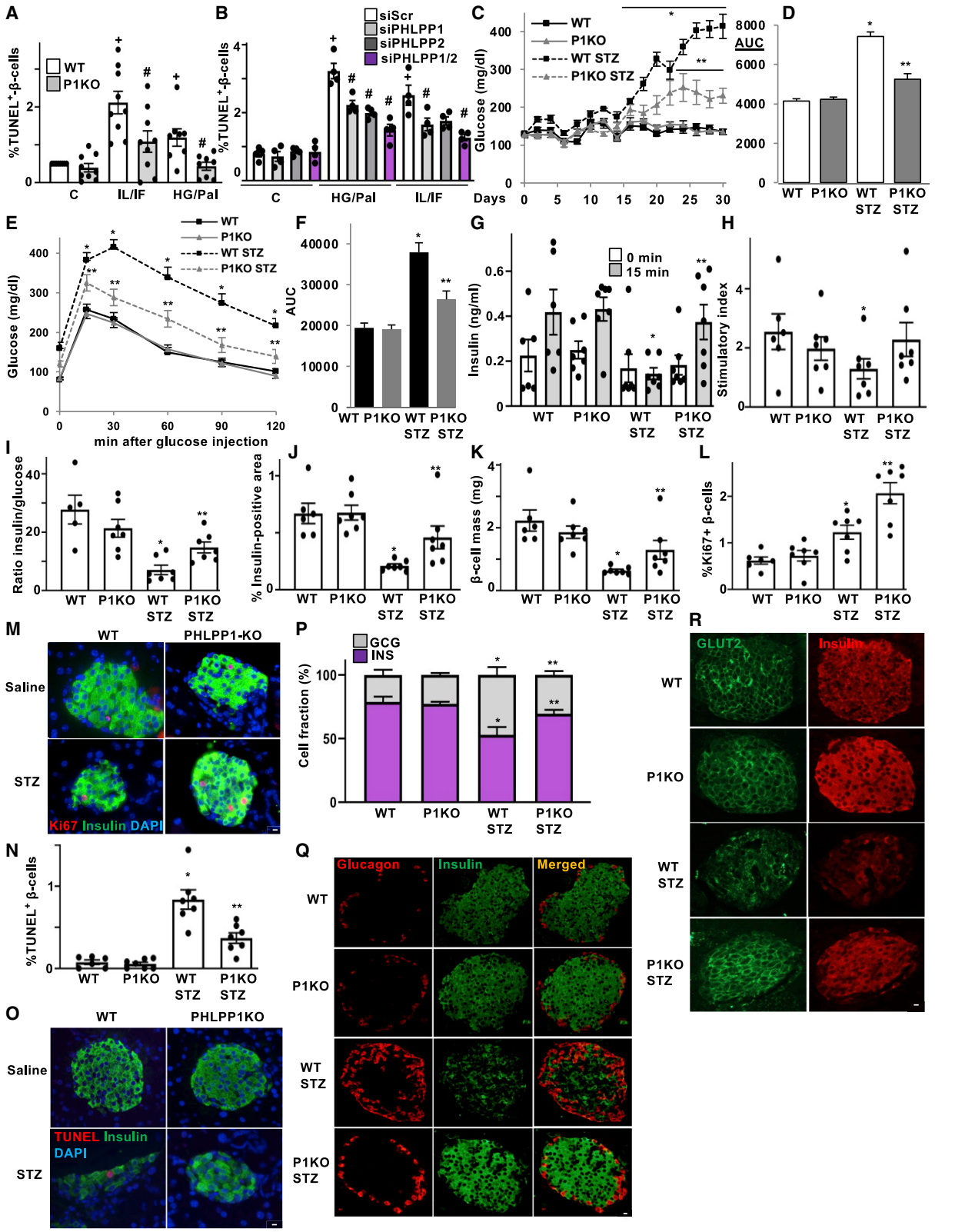
In summary, multiple gain- and loss-of-function experimental approaches targeting endogenous AKT and MST1 activities as well as PHLPP site-specific mutation analysis using phospho-mimetic mutants of AKT (AKT-S473D) and MST1 (MST1-T387E) showed that the critical kinases AKT and MST1 regulate the pro-apoptotic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

mTORC1 hyper-activation induces PHLPPs translation

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

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

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



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

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

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

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

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

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

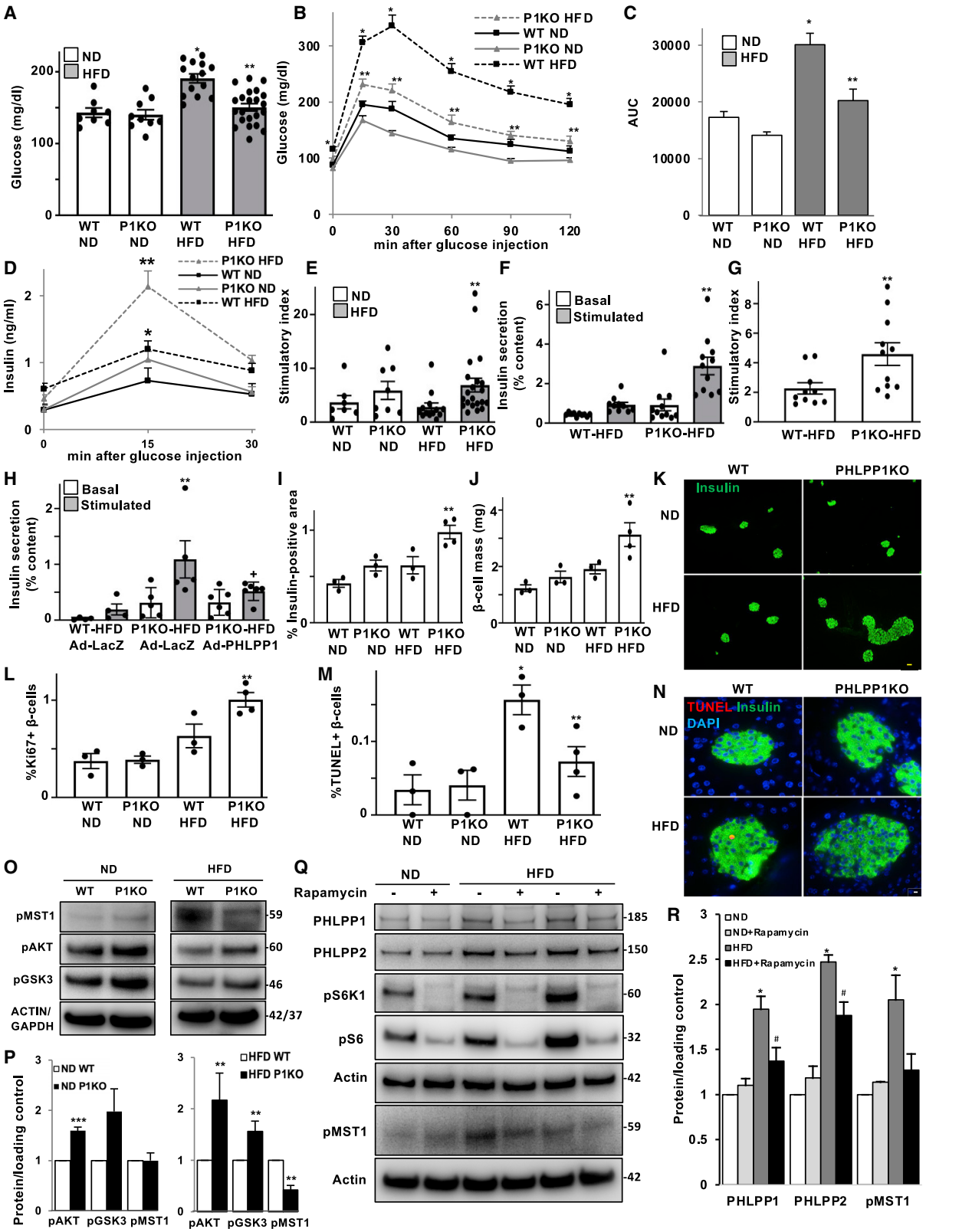
We next checked whether PHLPP1 deficiency could also restore the expression of several key markers of the glucose-sensing machinery as well as of insulin production. Immunostaining of pancreatic sections from STZ-treated mice showed a profound loss in both nuclear PDX1- and NKX6.1-positive cells. Many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

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

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

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

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



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

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

PHLPP1 deletion protects from HFD-induced diabetes

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

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

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

Figure 5. PHLPP1 deletion protects from HFD-induced diabetes

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

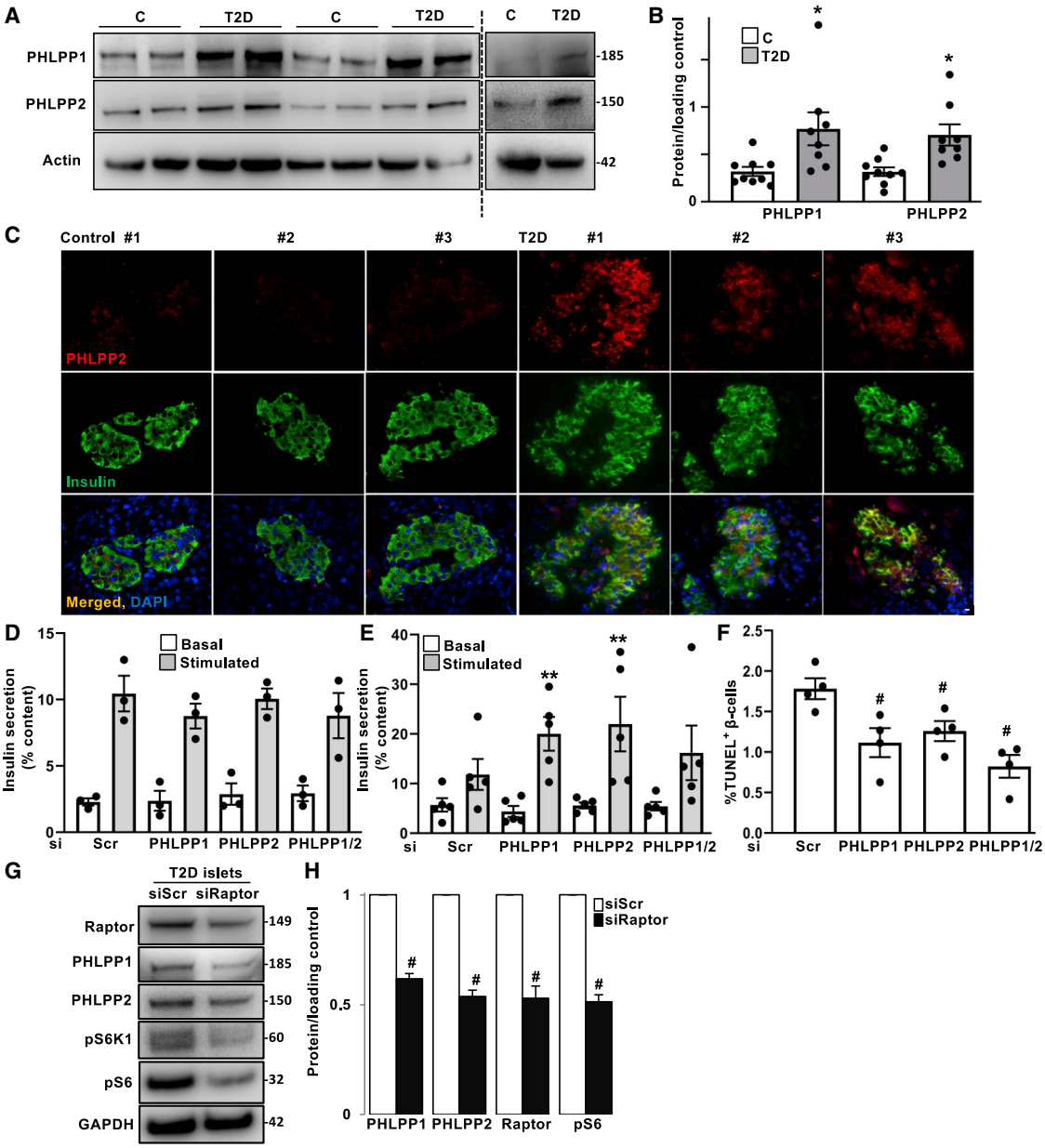

Figure 6. Genetic inhibition of PHLPP1/2 improved insulin secretion and β-cell survival in human islets from patients with T2D

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

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

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

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

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

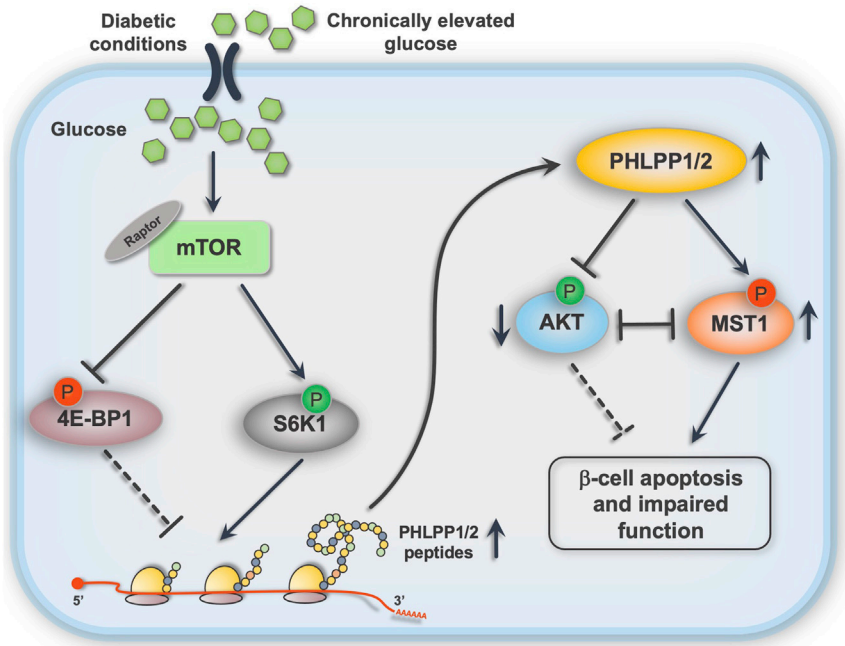

Figure 7. PHLPPing mTORC1 toward β-cell failure: Graphical summary of the results

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

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

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

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

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

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

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

Data and materials availability

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

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

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

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

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

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

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

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

RESOURCE AVAILABILITY

Lead contact

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

Materials availability

This study did not generate new unique reagents.

Data and code availability

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

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Cell culture, treatment and islet isolation

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

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

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

Animals

For multiple low dose streptozotocin (MLD-STZ) experiments, 8- to 10-week old male PHLPP1-KO mice (RRID: MGI:5795609; generously provided by Alexandra Newton, UCSD) (Masubuchi et al., 2010) on a C57BL/6J genetic background and their WT littermates were injected with multiple low-dose STZ (40 mg/kg body weight), freshly dissolved in 50 mM sodium citrate buffer, for five consecutive days. For the high-fat, high-sucrose diet (HFD) experiments, 8-week-old male WT and PHLPP1-KO mice were fed a normal diet (ND, Harlan Teklad Rodent Diet 8604, containing 12.2, 57.6 and 30.2% calories from fat, carbohydrate and protein, respectively) or a HFD (Surwit Research Diets, New Brunswick, NJ, containing 58, 26 and 16% calories from fat, carbohydrate and protein, respectively) for 17 weeks. For both groups, random blood was obtained from the tail vein of nonfasted mice, and glucose was measured using a Glucometer (FreeStyle; Abbott, IL, USA). Heterozygous leptin receptor deficient mice on the C57BLKS/J background ($Lepr^{db/+}$, $db/^{+}$) were purchased from Jackson Laboratory, ME, USA. By breeding of these mice, we obtained diabetic $Lepr^{db/db}$ (db/db) as well as non-diabetic heterozygous $Lepr^{db/+}$ (db/+) mice.

All mice used in the experiments were housed in a temperature-controlled room with a 12-h light-dark cycle and were allowed free access to food and water in agreement with NIH animal care guidelines, §8 German animal protection law, German animal welfare legislation and with the guidelines of the Society of Laboratory Animals (GV-SOLAS) and the Federation of Laboratory Animal Science Associations (FELASA).

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

METHOD DETAILS

Glucose and insulin tolerance tests and insulin secretion

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

Plasmids and siRNAs

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

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

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

Transfection

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

In vivo nucleic acid delivery

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

Adenovirus transduction

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

Glucose-stimulated insulin secretion (GSIS)

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

Immunohistochemistry

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

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

Morphometric analysis

For morphometric data, ten sections (spanning the width of the pancreas) per mouse were analyzed. Pancreatic tissue area and insulin-positive area (VECTASTAIN ABC Kit; Vector Labs, USA) were determined by computer-assisted measurements by using a Nikon MEA53200 (Nikon GmbH, Germany) microscope, and images were acquired by using NIS-Elements software from Nikon. Mean percent β-cell fraction per pancreas was calculated as the ratio of insulin-positive and whole pancreatic tissue area. Pancreatic β-cell mass was obtained by multiplying the β-cell fraction by the weight of the pancreas (Ardestani et al., 2014).

Western blot analysis

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

Protein degradation analysis

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

qPCR analysis

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

Translatome analysis

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

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

QUANTIFICATION AND STATISTICAL ANALYSIS

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

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

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My contribution:
Performed mouse islet isolations.

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

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ABSTRACT

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

1. Introduction

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

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

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genes that otherwise would compromise its functionality, for example by including alternative metabolic pathways. Over 60 of such "disallowed" genes that are specifically repressed in β-cells in a systematic way have been identified through genome-wide mRNA expression analyses [8–13]. De-repression of such disallowed genes could be connected to the impairment of β-cell function in diabetes [10,11,13–15].

Lactate dehydrogenase (LDH) is a metabolic enzyme that catalyzes the inter-conversion of pyruvate and lactate. Its isoform LDHA, which preferentially turns pyruvate into lactate, is such disallowed gene, highly repressed in pancreatic islets/β-cells. In addition to LDHA, the monocarboxylate transporter 1 (MCT1), a membrane protein that facilitates the transport of lactate and pyruvate across the plasma membrane, is also repressed in α-as well as β-cells [16]. The reason for the low expression of LDHA and MCT1 -as protective mechanism-is to prevent an inappropriate insulin release, triggered by circulating pyruvate or lactate, for example during exercise, which could generate hyperinsulinemia and result in hypoglycemia [11]. Importantly, previous studies have reported upregulation of LDHA in islets/β-cells from several rodent models of diabetes, either induced by partial pancreatectomy [15,17], in the Goto-Kakizaki (GK) [18–20], Zucker diabetic fatty (ZDF) [21] and human IAPP (HIP) transgenic rats [22] as well as in obese diabetic mice and in chronically high glucose exposed rat INS-1 β-cells [23]. Consistently, mouse senescent β-cells, which have been associated with impaired glucose tolerance and diabetes, also presented elevated LDHA expression levels [24]. Also, forced overexpression of LDHA has been associated with impaired glucose-stimulated insulin secretion (GSIS), a decrease in glucose oxidation rate or unusual insulin secretion stimulated by pyruvate and lactate in pancreatic rodent β-cell lines Min6 or INS-1 [25–28]. Altogether, these studies suggest that the LDHA expression level plays a critical role in the correct channeling of pyruvate into mitochondrial metabolism and overall, a proper insulin secretion, specifically stimulated by glucose.

Although repression of LDHA has been described as key β-cell signature in rodent islets/β-cells, little is known in human islet cells and whether they share a similar LDHA de-repression response documented in mouse models of diabetes. In the present study, we performed multi-approach analyses of LDHA expression in human islet α- and β-cells under normal physiological conditions and in metabolically stressed human islets and in individuals with T2D. We also addressed the impact of pharmacological inhibition of LDHA on insulin and glucagon secretion in human islets.

2. Materials and methods

2.1. Islet isolation, culture, and treatment

Human pancreatic islets isolated from pancreases of nondiabetic donors were provided by the UW Health Transplant Center (University of Wisconsin), the Translational Research Laboratory for Diabetes (University of Lille), the Alberta IsletCore (University of Alberta), the San Raffaele Diabetes Research Institute (ECIT, Milan), the Laboratory for Diabetes Cell Therapy (Plateforme de Recherche Ilots Montpellier Sud (PRIMS)) and the Endocrine surgery, kidney and pancreatic transplantation unit (CHEX, UCLouvain). Islets were cultured in non-coated petri dishes (#628161, Greiner Bio One) in RPMI 1640 (#11879–020, Gibco) at 5 mM glucose (UCLouvain) or on Biocoat Collagen I coated dishes (#356400, Corning, ME, USA) in full CMRL medium (Invitrogen) at 5.5 mM glucose (Uni Bremen) during 1–2 days for recovery and then exposed to increased glucose (22.2 mM) or physiological glucose (5.5 mM; control) for 24–72 h at 5% CO_2 and 37 °C. For glucose-stimulated insulin secretion (GSIS) or glucose inhibited glucagon secretion assays, human islets were additionally cultured with or without (control) 10 or 20 μM LDHA-selective inhibitor GSK 2837808 A (LDHAi; Tocris Bioscence, Bristol, UK) for 48 h on Collagen I coated plates. The inhibitor was added to the medium 1 h before glucose supplement. Ethical approval for the use of human islets had been granted by the Ethics Committee of the University of Bremen. The study complied with all relevant ethical regulations for work with human cells for research purposes and were performed in agreement with the local ethic committees and the institutional ethical committee of the French Agence de la Biomédecine (DC Nos. 2014–2473 and 2016–2716). Informed consent was obtained from all human islet donors' relatives. Organ donors are not identifiable and anonymous, such approved experiments using human islet cells for research is covered by the NIH Exemption 4 (Regulation PHS 398). Human islets were distributed by the two JDRF and NIHsupported approved coordination programs in Europe (Islet for Basic Research program; European Consortium for Islet Transplantation ECIT) and in the US(Integrated Islet Distribution Program IIDP) [29].

2.2. Fluorescence-activated cell sorting for gene expression measurements

Islets were trypsinized for dissociation into single cells. Dispersed cells were washed, filtered, and resuspended in a staining buffer (PBS, 1% BSA, 1 mM EDTA). Cells were stained in the dark using a combination of previously described antibodies [30] and sorted with a FACSAriaIII. The gating strategy involved exclusion of leukocytes ($CD45^+$), acinar/ductal cells ($CD44^+$), hematopoietic stem cells/endothelial cells ($CD34^+$), and acinar/ductal cells ($CD24^+$) from endocrine pan-islets cell (HPi 2^+), and a selective sorting of α- ($TM4SF4^+$) and β-cells ($CD9^+$).

2.3. Immunoblot analysis

After medium removal, human islets were washed twice with PBS and lysed with RIPA lysis buffer containing Protease and Phosphatase Inhibitors (Pierce, Rockford, IL, USA). Following the freeze-thaw cycles, the samples were incubated on ice for 30 min with intermittent vortexing. The lysate was centrifuged at 16000×*g* for 20 min at 4 °C and the clear supernatant containing the extracted protein was kept at −80 °C until needed. The protein concentrations were determined using the BCA Protein Assay Kit (Pierce). Protein samples were fractionated by NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electrically transferred into PVDF membranes. Membranes were then blocked in 2.5% non-fat dry milk (Cell signaling technology; CST) and 2.5% BSA (Sigma) for 1 h at room temperature and incubated overnight at 4 °C with the following antibodies: rabbit *anti*-LDHA (#2012) and rabbit anti-tubulin (#2146) from CST. Primary antibodies were followed by horseradish-peroxidase-linked anti-rabbit IgG secondary antibody (Jackson). All primary antibodies were used at 1:1000 dilution in Tris-buffered saline plus Tween-20 (TBS-T) containing 5% BSA. Membrane was developed using chemiluminescence assay system (Immobilon®, Millipore) and analyzed using the VisionWorksLS image acquisition and analysis software (UVP BioImaging Systems, Upland, CA, USA).

2.4. RNA extraction, cDNA synthesis and RT-PCR analysis

Total RNA was isolated from cultured human islets using TriFast™ (peqGOLD; Peqlab) or TriPure (Roche) for FAC-sorted cells according to the manufacturer's instructions. 500 ng to 1 μg of RNA were reverse transcribed using the RevertAid RT Reverse Transcription Kit (ThermoFisher) according to the manufacturer's

protocol, including removal of genomic DNA with DNase I prior to reverse transcription. Quantitative RT-PCR was performed as previously described [31]. StepOne Real-Time PCR system (Applied Biosystems, CA, USA) or CFX96 Real Time System (BioRad, CA, USA) with TaqMan® Fast Universal PCR Master Mix for TaqMan assays (Applied Biosystems) were used for analysis. TaqMan® Gene Expression Assays were used for human *LDH-A* (#Hs01378790_g1), *PPiA* (#Hs99999904_m1), *ACTB* (#Hs01060665_g1), *GCG* (#Hs01031536_m1), and *INS* (#Hs02741908_m1). qPCR was performed and analyzed by the Applied Biosystems StepOne or the BioRad CFX96 Real-Time Systems. The ΔΔCT or ΔCT methods were used to analyze the relative changes in gene expression.

2.5. Lactate assay

At the end of the incubation periods, culture media collected from human islets left untreated or treated with high glucose (22.2 mM) and their corresponding control culture media were subjected to deproteinization using 10kD spin columns (#ab93349 Abcam, Cambridge, UK), in order to minimize the interference of islet-released LDH into the media which could degrade lactate during the lactate assay and kept at −80 °C before analysis. Lactate concentration was determined by using a coupled enzymatic assay containing lactate dehydrogenase (LDH) and glutamate pyruvate transaminase (GPT; both Roche Diagnostics, Mannheim, Germany) as previously described in detail [32]. Briefly, aliquote volumes (10 μL or 20 μL) of the media were diluted with pure water to 180 μL in a well of a microtiter plate. To each well, 180 μL of a freshly prepared lactate reaction mixture (5.6 mM NAD^+, 3.89 U/mL GPT and 39.7 U/mL LDH in 0.5 M glutamate/NaOH buffer, pH 8.9) was added and the microtiter plate was incubated in a humidified atmosphere of an incubator at 37 °C for 90 min before the absorbance of the generated NADH at 340 nm was determined. Due to the 1:1 stoichiometry between lactate oxidation and NADH formation in this assay, the lactate concentration can be calculated by the Lambert-Beer law from the absorbance of NADH using the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. The basal lactate content of media that had no contact with islets (1.5 mM) was substracted from the media that had been harvested from the islets to determine the concentration of lactate released from the cells during the 72 h of incubation.

2.6. Immunostaining

Human pancreatic sections from nondiabetic and T2D human donors were provided from the National Disease Research Interchange (NDRI). PFA-fixed paraffin-embedded pancreatic sections and bouin-fixed human islets exposed to physiological (5.5 mM) or increased glucose (22.2 mM) were deparaffinized, rehydrated as described before [31] and incubated overnight at 4 °C with LDHA rabbit polyclonal antibody (#ab125683; Abcam) or for 2 h at room temperature with guinea pig anti-insulin (#A0546; Dako) or mouse anti-glucagon (#ab10988; Abcam), followed by fluorescein isothiocyanate (FITC)- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, PA, USA). The slides were mounted using mounting medium with DAPI (Vectashield®, Vector Labs). Immunostaining was analyzed using a Nikon MEA53200 (Nikon, Dusseldorf, Germany) microscope, and images were obtained using NIS-Elements imaging software version 3.22.11 (Nikon) and overlays created using ImageJ.

2.7. Insulin and glucagon secretion

Human islets (20–30 islets/dish) cultured on collagen-coated plates and treated with the LDHA-selective inhibitor were used for a glucose-stimulated insulin secretion assay performed as described before [31] or glucose inhibition of glucagon secretion. Islets were washed with PBS and preincubated with Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM (insulin) or 1 mM glucose (glucagon) at 37 °C for 30 min followed by fresh KRB containing 2.8 mM (insulin; basal) or 1 mM glucose (glucagon) for 1 h and additional 1 h in KRB containing 16.7 mM (insulin; stimulated) or 20 mM glucose (glucagon). Islets were washed with PBS and lysed with RIPA buffer for measuring total insulin or glucagon content. Insulin levels were detected by human insulin ELISA (ALPCO, Diagnostics). Glucagon was determined by the MSD Metabolic Assays/Human Glucagon Kit (#K151HCC-1; Meso Scale, Gaithersburg, MD, USA). Secreted insulin or glucagon was normalized to insulin or glucagon content, respectively.

2.8. Statistical analysis

The data are given as means ± SEM. To identify statistically significant differences, two-tailed student's t-test was conducted when data of at least 3 independent experiments were available. P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. LDHA is enriched in human pancreatic alpha cells

Due to the multi cell-type composition as well as cellular heterogeneity of human pancreatic islets, targeted cell-based analysis of the transcriptome using RNA-seq provides a valuable resource for the islet research community [33]. Bramswig et al. [34] previously established RNA-seq analysis to determine the transcriptional profiles of sorted α- and β-cell populations. Based on their cluster analysis across different cell types, *LDHA* is expressed nearly six times more in mature human α-cells compared to β-cells (Fig. 1A). To assess the developmental changes in LDHA expression in human islets, we analyzed LDHA expression in the RNA-seq dataset derived from purified fetal as well as adult α- and β-cells [35]. We found that fetal human α-cells expressed 1.5-fold higher *LDHA* levels compared to β-cells. However, while stable in α-cells at the adult stage, the expression levels of LDHA were dramatically reduced in human β-cells, *LDHA* was 15-fold higher expressed in α-cells in comparison to β-cells (Fig. 1B). Thus, the repression of LDHA in β-cells seems to occur from fetal-to adult development, and it is inversely correlated with β-cell maturation and regulated insulin secretion. To further support the α-cell enriched *LDHA* expression levels, we used several independent recent single-cell RNA-seq (scRNA-seq) datasets of human islets [36–39]. Analysis of such data across different studies confirmed that *LDHA* is enriched in human α-cells, compared to β-cells (Fig. 1C). Thus, an overall low expression of *LDHA* in islets can be explained by the lower percentage of α-cells present in islets, compared to β-cells and the strong repression of LDHA within β-cells.

To corroborate the results from the scRNA-seq data, we performed qPCR analysis on sorted α- and β-cells from five different human islet preparations. *LDHA* mRNA expression was higher in α-cells than β-cells in 4 out 5 analyzed human islet isolations (Fig. 1D). The two endocrine populations were validated by insulin and glucagon mRNA, which were almost exclusively expressed in β-cell and α-cell fractions, respectively, as shown before [30]; (Fig. 1E and F). Additionally, the cellular source of LDHA protein expression in human pancreatic islets was investigated through double-immunostaining of pancreatic autopsy samples from patients with T2D. In line with gene expression data, LDHA immunodetection revealed a strong colocalization of LDHA with glucagon-positive α-cells but not insulin-positive β-cells. Altogether, these

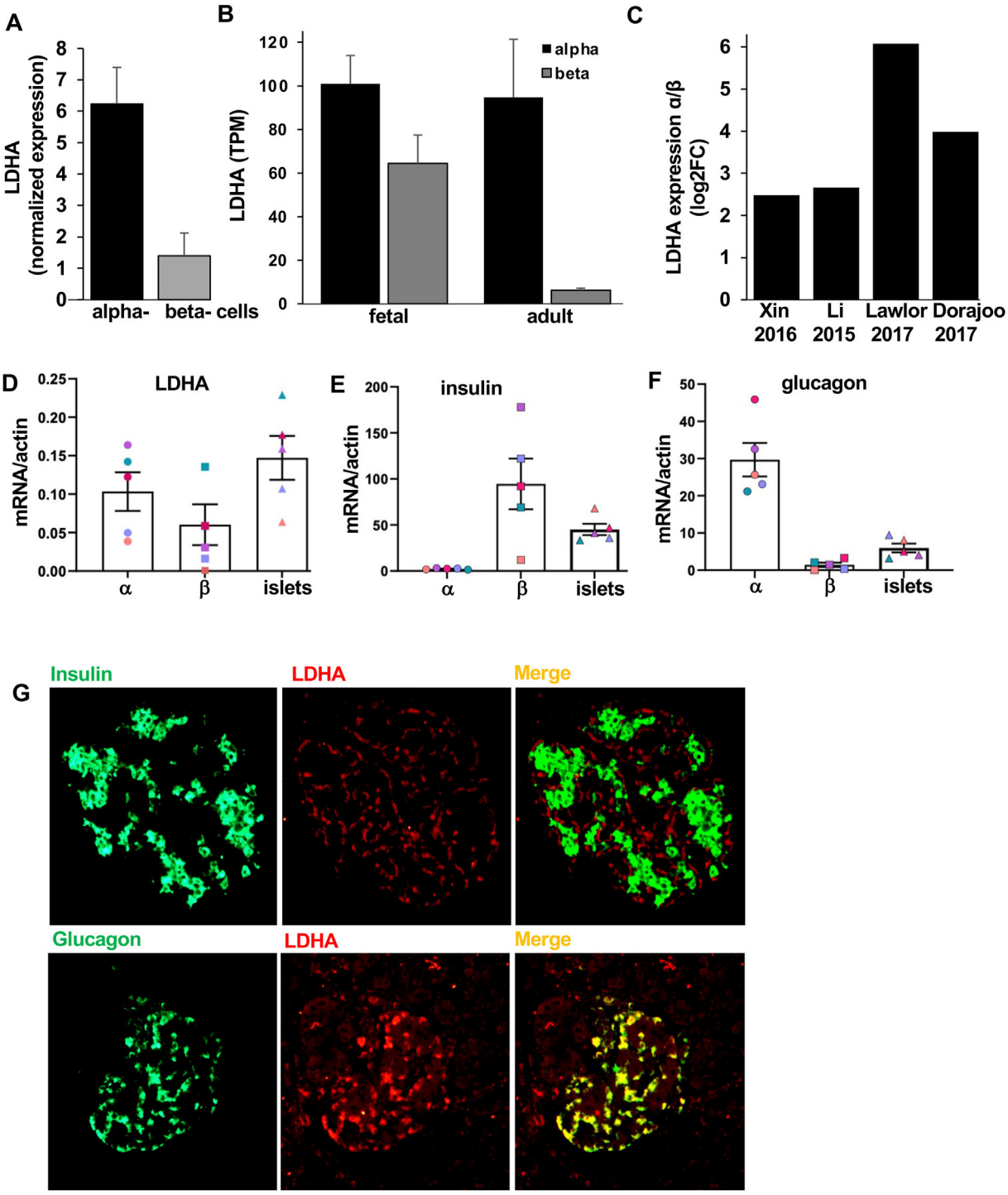

Fig. 1. LDHA is predominantly expressed in islet human alpha cells. LDHA transcript expression obtained from public available RNA-seq data sets **(A)** of sorted human α- or β-cells obtained from the analysis from Bramswig et al., **(B)** of sorted human fetal as well as adult α- or β-cells from Blodget et al. and **(C)** of human α- and β-cells expressed as log2FC α/β ratio from Xin et al., Li et al., Lawlor et al. and Dorajoo et al. **(D–F)** LDHA, insulin, glucagon and gene expression in human FAC-sorted α- and β-cells and pancreatic islets normalized to actin (n = 5 different human islet isolations from different donors). **(E,F)** Validation of the population purity through insulin and glucagon expression had been previously performed in 4 out of 5 isolations and thus, results from Fig. 5J,K of our previous publication [30] are included in these panels. **(G)** Representative double-stainings for insulin (green; upper panel), or glucagon (green; lower panel), and LDHA (red) are shown from human pancreatic sections from autopsy from patients with T2D (n = 3). Data are expressed as means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

data show that the human α-cell, but not the β-cell is the major source of LDHA expression in the human islets.

3.2. LDHA is upregulated in human islets in T2D

To investigate whether LDHA is upregulated in metabolically stressed human islets, they were exposed to high glucose concentrations for different periods of time. Notably, upregulation of *LDHA*

mRNA was evident under high glucose at 24 or 72 h compared to control (Fig. 2A). Also, human islets exposed to high glucose for 72 h exhibited significantly increased LDHA protein levels (Fig. 2B and C). LDHA is the isoform that has been shown to preferentially catalyze the conversion of pyruvate into lactate [40]. Consistently, the levels of lactate released by human islets cultured under high glucose were significantly higher than the levels from the control, confirming an increased activity of LDHA in human islets under glucotoxic condition (Fig. 2D). Such marked upregulation of both LDHA mRNA and protein observed under hyperglycemia in human islets supports the existing evidence of LDHA "derepression" as possible consequence of hyperglycemia in pancreatic islets [11,15,17].

Considering the heterogeneous composition of the islets in the pancreas, it is important to determine a cellular source of LDHA upregulation and its correlation to T2D. Bouin-fixed isolated human islets sections exposed to physiological (5.5 mM; control) or increased glucose (22.2 mM) were double immunostained for LDHA and glucagon. Again, LDHA colocalized with glucagon-expressing α-cells and was upregulated under glucotoxic conditions (Fig. 2E) confirming our expression data from bulk human islets. This suggests that basal as well as glucose-induced LDHA expression mainly occurred in α-cells. Pancreatic autopsy samples from T2D patients and controls were also double-stained to check whether α-cells from donors with established T2D also show LDHA upregulation. Also here, LDHA was found highly colocalized with glucagon-expressing α-cells and was markedly upregulated in the T2D pancreatic sections as compared to nondiabetic-control pancreases (Fig. 2F). Consistent with our finding, a study carried out by Lawlor et al. [37] has recently shown an upregulation of *LDHA* mRNA levels in human α-cells in T2D compared to control. Also, transcriptome profiling of a β-cell enriched fraction obtained by laser capture microdissection from individuals with T2D showed an increase of LDHA expression levels [41]; however, a purified α-cell fraction was not investigated in this work.

Similar to α-cell dysfunction in T2D [1,5], glucagon secretion is noticeably impaired in type 1 diabetes (T1D), which contributes to the susceptibility of patients to hypoglycemia [42,43]. Importantly, single-cell transcriptomes from cryo-preserved human islets isolated from T1D patients showed an elevated expression of *LDHA* transcript in α-cells compared to controls matched for BMI, age, sex, and storage time [44] suggesting a similar pathologic response might exist in the human islet α-cells under hyperglycemic T1D conditions.

Altogether, these findings indicate that upregulation of LDHA in stressed human islets *ex vivo* as well as in human T2D pancreatic islets occurred mainly in α-cells rather than in β-cells.

3.3. Impact of LDHA inhibition on the human islet hormones secretion

It has been proposed that impaired insulin secretion observed under hyperglycemic conditions is correlated with a metabolic shift involving upregulation of LDHA [19,20]. Having confirmed the upregulation of LDHA in diabetic human islets, the impact of chemical inhibition of LDHA on islet function was investigated in human islets exposed to chronically elevated glucose. The selective LDHA inhibitor GSK2837808 A (LDHAi) has previously successfully been used to target upregulated glycolysis as a metabolic hallmark of cancer cells [45–48]. LDHAi clearly decreases lactate release and increases glucose consumption in cancer cells [47]. Isolated human islets were exposed to 10 or 20 μM of LDHAi and then cultured with increased glucose concentrations for 48 h. Chronic high glucose exposure strongly abolished glucose-induced insulin secretion (Fig. 3A and B). Notably, the high glucose treated human islets presented a clear and robust increase in the basal insulin secretion phenocopying basal hyperinsulinemia triggered by exhausted dysregulated β-cells as a well-accepted feature of obese prediabetic as well as obese T2D patients [49,50].

LDHA inhibition by LDHAi, at 10 μM, increased glucose stimulated insulin secretion under physiological glucose concentrations and also revealed a tendency of increased glucose stimulated insulin secretion in islets under glucotoxic condition (Fig. 3A), but it did otherwise not show any significant impact on insulin secretion and had no effect on the stimulatory index on human islets exposed to high glucose (Fig. 3B).

The regulation of glucagon secretion is complex, multifactorial and includes cell intrinsic and paracrine elements [1,5,51]. As LDHA is enriched and upregulated in α-cells in T2D and glucagon physiologically released from α-cells potently participates in the regulation of glycemia, LDHAi was used to investigate its impact on glucagon release in human islets exposed to hyperglycemia. LDHAi showed a tendency of increased glucagon secretion at 1 mM low glucose, both in islets chronically exposed to physiological as well as elevated glucose, however, glucagon secretion showed a high variability and neither of the effects could reach significance in our setting suggesting that changes in LDHA might be dispensable for glucagon secretion in human islets.

In summary we show that LDHA belongs to the group of "disallowed genes" in human β-cells; it is predominantly expressed in α-cells and its level is highly elevated in islets/α-cells in metabolically stressed human islets as well as in pancreases from donors with T2D.

Previous work has suggested a link between de-repressed LDHA and β-cell dysfunction in T2D [11,15,17]. Although the mechanisms underlying this connection were not fully elucidated, a number of attempts to block the activity of LDHA in islets/β-cells have been carried out. Initially, Sasaki et al. showed that neutralizing diabetes-associated elevation of reactive oxygen species (ROS) in rat diabetic GK islets by antioxidants counteracts the upregulation of the major metabolic transcription factor HIF1α and its downstream target genes including LDHA. Consequently, lactate production decreased, whereas insulin secretion improved [19]. Also, *in vivo* treatment of obese diabetic *db/db* mice with the LDHA inhibitor oxamate, an analog of pyruvate showed a significant restoration in metabolic parameters such as fasted blood glucose, insulin sensitivity and insulin secretion as well as of pancreatic islets morphology [52]. The elevated glucose level has not only been associated with differential expression of LDHA but also with an overall alteration of the coordinated expression of genes involved in glycolytic and mitochondrial metabolism, generating a metabolic shift observed at an early stage of T2D [20]. In contrast and under physiological conditions, current data show that LDHB, the other LDH isoform, is highly expressed in human β-cells and has been acknowledged as a "β-cell signature gene" [37,39] that favors the internal lactate to pyruvate flux in order to preserve and maximize, when needed, mitochondrial transport, oxidative phosphorylation and subsequent insulin secretion. Thus, these studies suggest that elevated LDHA and/or potentially declined LDHB and subsequent lactate overproduction could potentially associate with T2D as it might compromise β-cell mitochondrial oxidative phosphorylation and subsequent insulin secretion by lowering the level of pyruvate and

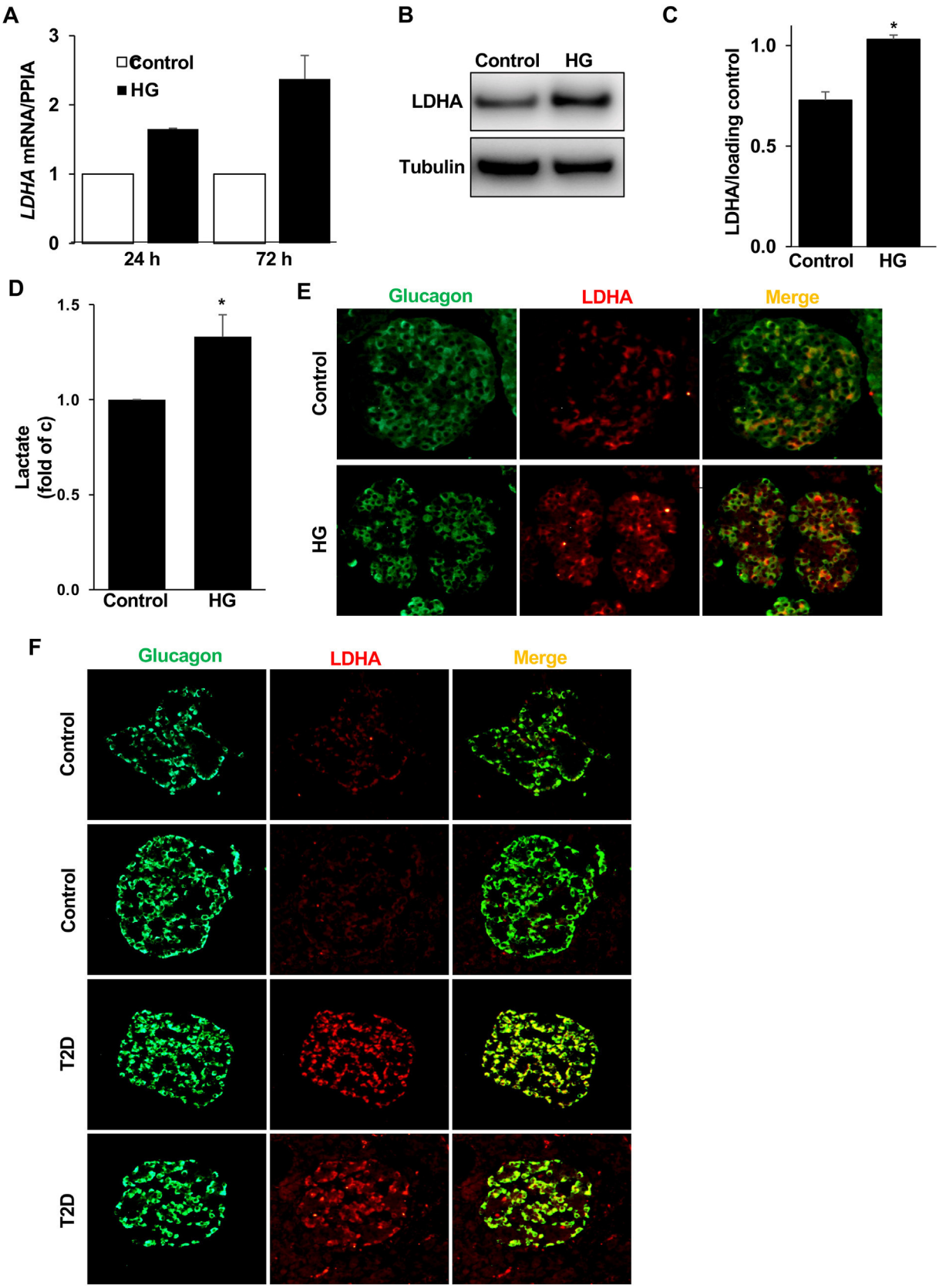

Fig. 2. LDHA is upregulated in human alpha cells. (A) qPCR for LDHA mRNA expression in isolated human islets treated with high glucose (HG; 22 mM) for 1 or 3 days normalized to *PPIA* (n = 2 different human islet isolations). **(B,C)** Representative Western blots **(B)** and quantitative densitometry analysis **(C)** of isolated human islets treated with high glucose (HG; 22 mM) for 3 days (n = 3 different human islet isolations). **(D)** Lactate levels in culture media collected from isolated human islets treated with high glucose (HG; 22 mM) for 3 days normalized to control conditions (n = 4 different human islet isolations from 4 different donors). **(E)** Representative double-stainings for LDHA (red) and glucagon (green) shown from isolated human islets left untreated (cont) or treated with high glucose (HG; 22 mM) for 3 days. **(F)** Two sets of representative double-stainings for LDHA (red) and

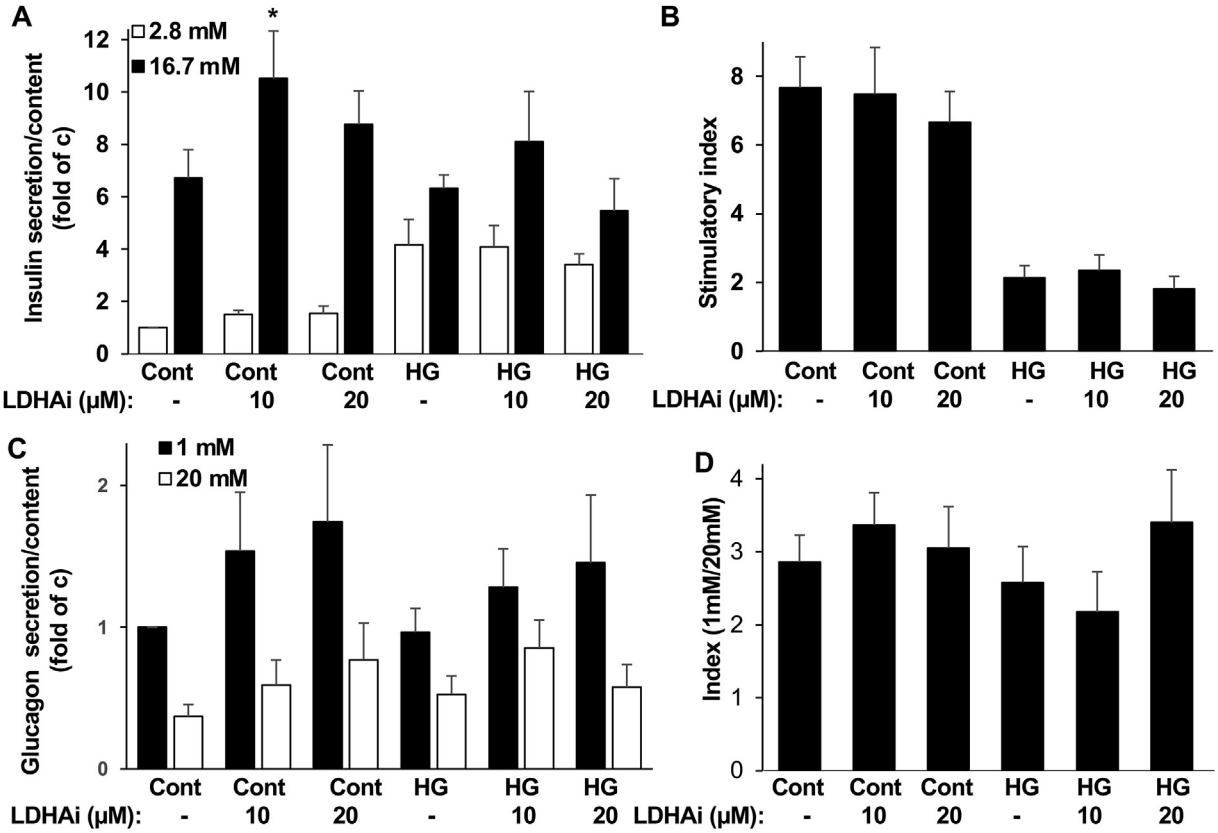

Fig. 3. Impact of LDHA inhibition on insulin and glucagon secretion. Isolated human islets treated with or without (control: cont) 10 or 20 μM LDHA inhibitor GSK2837808 A (LDHAi) 1 h before and during the 48 h exposure to physiological glucose (5.5 mM; cont) or to high glucose (22 mM; HG). Thereafter, **(A)** Insulin secretion was analyzed during 1-h incubation with 2.8 mM (basal) and 16.7 mM (stimulated) glucose normalized to insulin content, **(B)** the insulin stimulatory index denotes the ratio of secreted insulin during 1-h incubation with 16.7 mM to secreted insulin at 2.8 mM glucose. **(C)** In a parallel set of islets, glucagon secretion was analyzed during 1-h incubation with 1 mM and 20 mM glucose normalized to glucagon content, **(D)** the glucagon secretory index denotes the ratio of secreted glucagon during 1-h incubation with 1 mM to secreted glucagon at 20 mM glucose. A-D (n = 8–9; from 3 different human islet isolations). Data are expressed as means ± SEM. *p < 0.05 compared to untreated stimulated control.

reducing equivalents in mitochondria. It is important to note that the aforementioned studies tested LDHA inhibition in the rodent diabetic models but not in human islets. Our data show that LDHA inhibition moderately enhanced stimulated insulin secretion under physiological conditions but overall did not restore β-cell function or corrected glucagon secretion under high glucose conditions. However, in depth studies including a greater number of human islet preparations -as we were only able to perform the LDHA inhibition experiment in three independent isolations-as well as dynamic perfusion analysis of insulin and glucagon release *ex vivo* or *in vivo* is required for a further detailed evaluation of a possible causative role of LDHA in the islet secretory dysfunction and diabetes progression.

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Author contributions

PMS performed experiments, analyzed data and wrote the paper.

MK, EG, SG, BL, HL performed experiments, and analyzed data.

RD, PG, AW provided intellectual support and scientific material.

AA, KM designed experiments, analyzed data, supervised the project and wrote the paper.

Data availability

Raw Western Blots are included in this submission. All data are included in the manuscript and will be further made available upon request.

Declaration of competing interest

The authors declare no conflict of interests.

glucagon (green) are shown from human pancreatic sections from autopsy from nondiabetic controls (n = 3) or from donors with type 2 diabetes (T2D) (n = 3). Data are expressed as means ± SEM. *p < 0.05 compared to untreated control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.06.065.

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

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

Manuscript I: Enteroviral infections are not associated with Type 2 diabetes

Experimental concept and design:	ca. 20%
Experimental work and/or acquisition of (experimental) data:	ca. 50%
Data analysis and interpretation:	ca. 50%
Preparation of Figures and Tables:	ca. 20%
Drafting of the manuscript:	ca. 20%

Manuscript II: The Hippo terminal effector YAP boosts enterovirus replication in type 1 diabetes

Experimental concept and design:	ca. 20%
Experimental work and/or acquisition of (experimental) data:	ca. 40%
Data analysis and interpretation:	ca. 40%
Preparation of Figures and Tables:	ca. 40%
Drafting of the manuscript:	ca. 20%

Publication I: Enteroviruses and T1D: Is It the Virus, the Genes or Both which Cause T1D

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

Publications in the appendix

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

Experimental concept and design:	ca. 5%
Experimental work and/or acquisition of (experimental) data:	ca. 5%
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Drafting of the manuscript:	ca. 5%

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

Experimental concept and design:	ca. 5%
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