Environmental input to the pancreatic β-cells - the role of mechanosensitive and other ion channels

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Abstract

Compound input from genetic predisposition, environmental factors and lifestyle lead to β-cell dysfunction which initiates the development of type 2 diabetes. Understanding the linkage between the environmental input and gene regulatory pathways controlling β-cell function is key for developing novel therapies against T2D.

The pancreatic β-cell is controlled by ion channels. Voltage-gated Ca^{2+} channels (VGCC) regulate Ca^{2+} signaling and insulin secretion. They are assembled with pore-forming α1 subunits and auxiliary subunits (α2δ, β, γ). Very recently, the mechanosensitive channel Piezo1 was suggested as a stimulator of insulin secretion. Mechanotransduction transduces mechanical forces into intracellular signalings and affects various cellular processes, possibly also insulin secretion. Genetic predisposition controls the susceptibility for T2D. The transcription factor TCF7L2 harbors the strongest diabetes risk gene variant and controls gene networks in insulin processing and secretion. MafA is a β-cell maturation marker, its expression is tightly associated with the differentiation state of β-cells. However, the exact mechanism behind Piezo1 regulated insulin secretion and how Tcf7l2 and MafA affect ion channels remain unknown.

Results: PIEZO1 is significantly upregulated in islets from T2D donors and also under the conditions of developing diabetes. Hyperglycemia triggers translocation of Piezo1 into the nucleus and normoglycemia can reverse this abnormal distribution. Inhibition of Piezo1 by GsMTx4 reduces swelling/glucose-induced Ca^{2+} signaling, membrane depolarization and insulin secretion. Silencing of *Piezo1* reduces Ca^{2+} handling and impairs glucose-stimulated insulin secretion (GSIS) while yoda1, the specific activator of Piezo1 induces such responses. Piezo1 regulates abundant genes (most notably *Cartpt*). Next, we generated a β-cell specific *Piezo1* knockout mouse model and ablation of *Piezo1* in β-cells results in an age-dependent effect on glucose utilization and insulin secretion. *Piezo1* deletion strongly reduced glucose-stimulated electrical activity in β-cells. These results highlight Piezo1 as a key regulator of β-cell function *in vivo* and *in vitro*.

Tcf7l2 regulates both mRNA and protein levels of α2δ-1. Suppression of α2δ-1 reduces Ca^{2+} currents and glucose/depolarization-induced Ca^{2+} concentration which mimics the effect of silencing of *Tcf7l2*. Silencing of *Cacna2d1* impairs GSIS and overexpression of α2δ-1 improves it by α2δ-1 regulated Cav1.2 trafficking. Importantly, re-introducing α2δ-1 recovers the Tcf7l2-dependent impairment of Ca^{2+} signaling, but not the reduced insulin secretion. Taken together, these data demonstrate that α2δ-1 is the target of Tcf7l2 in controlling Ca^{2+}-signaling.

Cavγ4 is downregulated in islets from hyperglycemic human donors and T2D rodent models. Silencing of *Cacng4* inhibits Ca^{2+} influx and insulin secretion by suppressing the expression of L-type Ca^{2+} channels (Cav1.2 and 1.3). MafA regulates γ4 expression by directly binding to its promoter. Cavγ4 expression is also associated with β-cell differentiation state verified by testing the de-differentiation marker Aldh1a3. These findings demonstrate that γ4 is part of MafA mediated β-cell differentiation and suggest the potential role of γ4 for correcting β-cell dysfunction.

Conclusions: This thesis presents evidence for novel regulatory pathways involving mechanosensor Piezo1, Tcf7l2 and MafA controlled Cavα2δ-1 and γ4, respectively, for preserving β-cell function and normal insulin secretion. These findings update the current consensus model of Ca^{2+}-dependent insulin release. Mediating Piezo1 activity to optimize β-cell response to environmental input, recovering α2δ-1 or γ4 expression to restore β-cell function may also serve as new potential therapies to T2D.

Key words: T2D, pancreatic islets, mechanosensitive channels, Piezo1, β-cell specific Piezo1 knockout mouse, Tcf7l2, α2δ-1, MafA, γ4, β-cell function, insulin secretion, dedifferentiation, Ca^{2+} signaling, transcription factor

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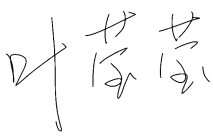
Signature  Date 2020-03-25

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To my family

Contents

Papers included in the thesis

I. **Ye Y**, Barghouth M, Wang Y, Luan C, Karagiannopoulos A, Jiang X, Krus U, Eliasson L, Rorsman P, Zhang E, Renström E, The mechanosensor Piezo1 mediates glucose sensing and insulin secretion in pancreatic β-cells. *Manuscript.*

II. **Ye Y**, Barghouth M, Wang Y, Fex M, Dou H, Eliasson L, Zhang E, Renström E. Beta-cell specific Piezo1 deficient mice reveal Piezo1 regulates glucose utilization and insulin secretion in rodent pancreas. *Manuscript.*

III. **Ye Y**, Barghouth M, Luan C, Kazima A, Zhou Y, Eliassona L, Zhang E, Hansson O, Thevenin T, Renström E (2020), The TCF7L2-dependent high-voltage activated calcium channel subunit α2δ1 controls calcium signaling in rodent pancreatic beta-cells. *Mol Cell Endocrinol*. 502: p. 110673.

IV. Luan C, **Ye Y**, Singh T, Barghouth M, Eliasson L, Artner I, Zhang E, Renström E (2019). The calcium channel subunit gamma-4 is regulated by MafA and necessary for pancreatic beta-cell specification. *Commun Biol*. 2: p. 106.

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Abbreviations

T1D	Type 1 diabetes
T2D	Type 2 diabetes
GDM	Gestational diabetes mellitus
$[Ca^{2+}]_i$	Free cytosolic Ca^{2+} concentration
GWAS	Genome-wide association analysis
TCF7l2	Transcription factor 7-like 2
ADRA2A	α2A-adrenergic receptor gene
eNOS	endothelial nitric oxide synthase
Glut 2	Glucose transporter 2
GCK	enzyme glucokinase
K_{ATP} channel	ATP-sensitive potassium channel
Sur	Sulfonylurea receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
GSIS	Glucose-stimulated insulin secretion
SENP1	deSUMOylating enzyme
TCA	Tricarboxylic acid cycle
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid
VRAC	Volume-regulated anion channel
Swell1	Leucine-rich repeat (LRR) containing protein
VGCC	Voltage-gated calcium channel
TRP channels	Transient Receptor Potential channels
TGH	Glycosylated hemoglobin
MS channels	Mechanosensitive ion channels
DHSt	Dehydrated hereditary stomatocytosis
VDCC	Voltage-dependent Ca^{2+} channels
DHPs	Dihydropyridines
PM	Plasma membrane
TARPs	Transmembrane AMPA receptor regulatory proteins
GK rats	Goto-Kakizaki rats
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
Vamp2	Vesicle-Associated Membrane Protein
TF	Transcriptional factors
Maf	Musculoaponeurotic fibrosarcoma oncogene family
PDX1	Pancreatic duodenal homeobox 1
IPF1	Insulin promoter factor 1
Ngn3	Neurogenin 3
TCF7L2	Transcription factor 7-like 2
GLP-1 and 2	Glucagon-like peptides
LSL	Lox-stop-lox
KO	Knockout

RIP-Cre^{+}	Rat insulin 2 gene promoter-driven Cre
Cre^{+}.P1$^{f/f}$	β-cell specific Piezo1 knockout mice
IPGTT	Intraperitoneal glucose tolerance test
RIA	Radioimmunoassay
RRP	Readily releasable granules
RNA-seq	RNA-sequencing
DZX	Diazoxide
GO	Gene Ontology
CART	Cocaine- and amphetamine-regulated transcript
HSIS	Hypotonicity-stimulated insulin secretion
GBP	Gabapentin
DRG	Dorsal root ganglion
Hap1	Huntingtin-associated protein 1
PKC	Protein kinase C
ER	Endoplasmic reticulum
MafA$^{\Delta\beta cell}$	β-cell specific MafA ablation in mice
Aldh1a3	Aldehyde dehydrogenase1A3
SPIONs	Superparamagnetic nanoparticles

Introduction

Diabetes Mellitus

Diabetes mellitus (hereafter referred to as 'diabetes') is a major health threat and one of the fastest increasing burdens to human health today. Nearly a half-billion (463 million) of people are estimated to live with diabetes today, and the number is expected to reach 700 million by 2045 [1].

Diabetes is defined as a chronically elevated blood glucose concentration, mainly caused by inadequate release of the glucose-lowering hormone insulin or inability of response to insulin in target cells (primarily in skeletal muscle, adipose tissue and liver) [1]. So far, Diabetes is presently divided into a few subtypes, the most common are type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes mellitus (GDM). T2D accounts for ~90% of all diabetes globally. The phenotype of T2D is less dramatic than that of T1D, it may even be completely without symptoms initially. As a result, as many as ~50% of the T2D population might remain undiagnosed until complications such as retinopathy, cardiovascular diseases, nephropathy, or neuropathy emerge [2, 3]. These resultant severe complications have a major impact on the quality of life and life expectancy. To prevent these, early diagnosis and care for all diabetic patients are crucial. Furthermore, T2D at an early stage can be reversed (e.g. by weight loss) while T2D with longer duration has permanent pancreatic cell changes (e.g. β-cells) and is difficult to return to normal [4]. By preventing or taking actions to reverse pancreatic β-cell changes, life-threatening symptoms can be delayed, or even prevented, by proper management of diabetes. What is the ideal treatment of diabetes? To achieve this, we need to further study and understand the functions of pancreatic β-cells in the pathogenesis of T2D.

Causes of Type 2 Diabetes

Pancreatic islets are clusters of endocrine cells scattered within the pancreas [5]. The islet contains five major endocrine cell types: β-cells (secreting insulin), α-cells (glucagon), δ-cells (somatostatin), pancreatic polypeptide (PP)-producing cells, and ε-cells (ghrelin). The pancreatic β-cells compose the majority of the islets. Loss of

function in pancreatic β cells in combination with insulin resistance result in persistent hyperglycemia and T2D [6, 7]. T2D patients, even in early disease stages, lose >80% of β-cell function measured by disposition index (insulin secretion/insulin resistance) [8]. This indicates that loss of β-cell function is an early event in the development of T2D [9]. Although insulin resistance attributes to T2D, overt diabetes only occurs in the presence of progressive β-cell dysfunction [10].

Pancreatic β-cell dysfunction results from a polygenic predisposition as well as environmental factors [1, 11]. Genome-wide association analysis (GWAS) has identified a plethora of genetic variants significantly associated with β-cell failure and T2D [12, 13]. For example, the strongest T2D risk gene candidate *TCF7L2* is related to impaired insulin production and release [14], the underlying mechanism will be detailed later. Elevated expression of human α2A-adrenergic receptor gene *ADRA2A* is tightly related to reduced insulin secretion [15]. The gene *KCNJ11* encoding ATP-sensitive K^+ channel Kir6.2 regulates the K^+ inward currents to depolarize β-cell membrane thus stimulates insulin granule exocytosis [16].

Moreover, long-term intake of high-calorie foods, lack of physical exercise lead to weight gain and result in insulin resistance. This leads to extra requirement of insulin, but if beyond the body's compensatory capacity, it also evokes glucotoxicity and lipotoxicity that accelerate β-cell failure [17]. So far, the pathogenesis of β-cell dysfunction has attracted enormous attention, but the causes of T2D are still not fully understood. Here, I will specifically introduce some aspects influencing β-cell functions that are highly related to T2D development.

Insulin synthesis

Insulin is necessary for life and the only hormone capable of lowering blood glucose. It was discovered by Frederick G Banting, Charles H Best and John James Rickard Macleod, purified by James B. Collipin in 1921 [18]. Insulin is a strongly conserved protein with 51 amino acids, encoded by the INS gene [19-21]. Preproinsulin is translated from its mRNA, cleavage of the N-terminal peptide yields a single chain of proinsulin, which contains an A-chain (21 amino acids long) and a B chain (30 amino acids long) connected via C-peptide. Mature insulin is formed after cleavage of C-peptide, the A and B chains are retained but connected by two disulfide bonds [21]. Both mature insulin and C-peptide are co-secreted in equimolar amounts from the β-cell secretory granules [20, 21]. Insulin has a half life of ~6 min while ~30 min for C-peptide, which makes the measurement of C-peptide more reliable as an assessment of insulin secretion [22]. C-peptide is found to stimulate Na^+/K^+-ATPase activity and endothelial nitric oxide synthase (eNOS) [23]. C-peptide improves erythrocyte deformability in T1D patients [24]. Appropriate administration of C-peptide in T1D patients results in improved

circulatory responses by increasing blood flow in skeletal muscle [25, 26], skin microvascular [27, 28] and kidney [29].

Insulin action

Insulin is essential for converting glucose into energy, promoting the storage and utilization of energy in the fasting and fed state, respectively [21]. The blood glucose level is controlled within a narrow range by exact regulation of insulin secretion by nervous and hormonal input, but primarily locally in the β-cell. Insulin is important for the metabolism of carbohydrates (blood glucose), fat (lipid storage), and also protein (branched-chain amino acids) [21]. Once insulin is secreted from the pancreatic β-cells upon stimulation of elevated serum glucose and enters the systemic circulation, a variety of actions are initiated by binding to the insulin receptors in target tissues [30]. The first target organ is the liver [31]. Insulin lowers blood glucose concentration by inhibiting hepatic glucose production (e.g. inhibit glycogenolysis and conversion of amino acids to glucose [21]) [32]. More than 50% of the insulin delivered to the liver is utilized and degraded [33], what remains after the first-pass clearance exits the liver and arrives at the heart via the vena cava venous circulation, is then distributed to the rest of the body following the arterial circulation. Insulin is also transported through the blood-brain barrier into the hypothalamus, hippocampus, and cerebral cortex where insulin receptors are broadly expressed and affect feeding behavior, body weight handling, etc [34]. Muscle and fat cells exposed to insulin accelerate glucose uptake by stimulating glucose transport, finally, insulin actions occur in the kidney [31].

Insulin Secretory Pathways

In pancreatic β-cells from healthy individuals, increased glucose stimulates insulin secretion via a triggering pathway (K_{ATP} channels closure, depolarization-triggered activation of voltage-gated Ca^{2+} channels, and rise in free cytosolic $[Ca^{2+}]_i$) and an amplifying pathway (enhancement of Ca^{2+} efficacy on insulin release). Furthermore, increasing pieces of evidence have shown the involvement of mechanosensitive ion channels (TRP channel superfamily, volume regulated anion channels, etc) in the regulation of insulin exocytosis, we name this the mechanosensing pathway. These pathways will be introduced in detail below.

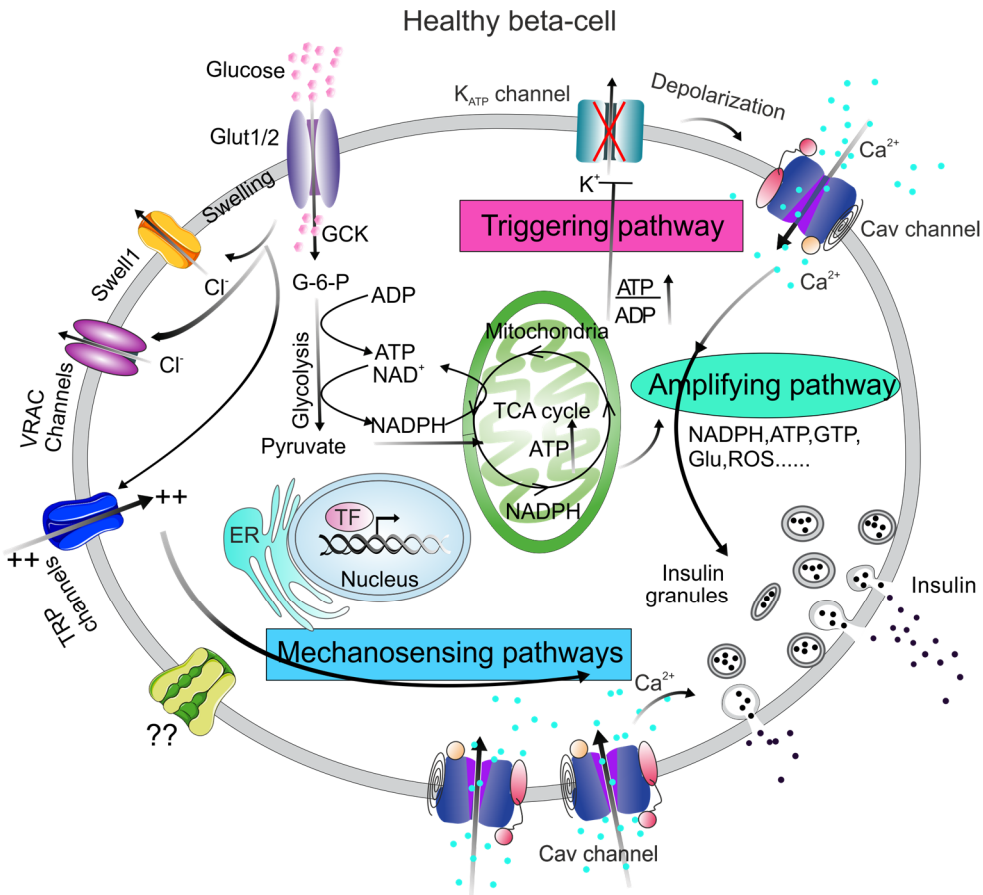

Figure 1 Brief summary of insulin secretion pathways including triggering pathway, amplifying pathway and possible mechano-sensing pathways.

Triggering Pathway

Elevated blood glucose (e.g. postprandial) is taken up by β-cells through glucose transporters, Glut 2 mainly in rodents, but Glut 1, 3 and 4 predominantly facilitate glucose entry in human β-cells [35-37]. Metabolism of entered glucose is initiated by the enzyme glucokinase (GCK) that catalyzes glucose into glucose 6 phosphate to generate ATP via glycolysis in the mitochondria, and this also causes a concomitant fall in MgADP [10, 38]. The increased ATP/ADP ratio closes ATP-sensitive potassium (K_{ATP}) channels, accumulation of K^+ results in less negative charge inside the cell, or depolarization, of the cell membrane [39]. This triggers voltage-gated calcium channels opening for calcium ion influx, the induced cytosolic Ca^{2+} ultimately stimulates the exocytosis of insulin granules docked at the plasma membrane [40]. Conversely, at the resting state, i.e. low plasma glucose,

the K_{ATP} channels are open and the membrane stays hyperpolarized due to continuous K^+ efflux, this inhibits electrical activities, prevents opening of calcium ion channels and insulin secretion [10].

Insulin secretion occurs in two phases: the first phase immediately responds to the increased glucose levels (reach the peak with 3-5 min) and lasts for ~10 min. This results from the exocytosis of predocked insulin granules in response to the elevation of Ca^{2+}, then it is followed by a long-term second phase of insulin secretion lasting for up to several hours, and that has been suggested to result from the time-consuming refilling of the releasable pool of insulin granules [41]. This physiological regulation of insulin secretion pathway has been regarded as a consensus model for decades, however, other pathways might also be involved [31, 42, 43], so is this the end of story?

Amplifying pathway

Glucose is the primary stimulator of insulin secretion, but its effect is not limited to increasing ATP concentrations, controlling Ca^{2+} signaling and inducing insulin secretion. The metabolic amplification of insulin exocytosis upon glucose metabolism is also a facet of its actions. Solid evidence has developed since 1992 and a brief summary of key findings is as follows: the K_{ATP} channel opener diazoxide [44] binds to sulfonylurea receptor Sur1 (a subunit to form K_{ATP} channel) [45], prevents a majority of the effects of glucose on β-cell membrane depolarization, augmentation of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) and insulin secretion. The application of glucose has a further augment of K^+-stimulated insulin secretion in the presence of diazoxide in rodent islets [46, 47]. Many other groups also extended this concept to human islets [48], and various insulin-secreting cell lines [49]. In contrast, when K_{ATP} channels are completely blocked by sulfonylureas, glucose still has the ability to increase insulin secretion even though the β-cell membrane is already depolarized and $[Ca^{2+}]_i$ is raised [50, 51]. Mice without functional K_{ATP} channels (Sur1 knockout mice or Kir6.2 deficient mice), exhibit a relatively high "basal" $[Ca^{2+}]_i$ and insulin secretion rate, but a transient increase in $[Ca^{2+}]_i$ following high glucose treatment and sustained activation of insulin secretion was unsuspectedly found. This confirms the involvement of an additional amplifying pathway [52, 53]. Importantly, insulin stimulation by this pathway can be completely inhibited by $[Ca^{2+}]_i$ influx omission [54, 55], which means that the amplifying pathway requires an initial increase in $[Ca^{2+}]_i$ for triggering insulin release. It finally turned out that glucose has an additional effect in the amplifying pathway which is independent of K_{ATP} channels' actions and that augments the magnitude of insulin secretion. This metabolic amplification is fast and affects both the first and second phases of insulin secretion [56]. However, the exact mechanisms behind the amplifying pathway are still elusive.

Previous reports point to the importance of NADPH in the influence of insulin secretion which can be one of the regulating factors in the amplifying pathway [57]. The disturbance of the pentose phosphate pathway producing NADPH has negative effects on glucose-stimulated insulin secretion (GSIS) [58-60]. Novel techniques have been developed to measure NADPH production and its functions in the amplifying pathway [61-63], which have been improved to uncover previously little understood regulators of the amplifying pathway. Multiple studies support that the deSUMOylating enzyme SENP1 is also a contributor to the amplifying pathway [64, 65]. Pyruvate is generated by glycolysis and is transported into the mitochondria, and its metabolism is confirmed to be involved in the metabolic amplification [66], where half of the pyruvate is utilized to regenerate oxaloacetic acid (OAA) entering the tricarboxylic acid cycle (TCA) for ATP synthesis, while another half is metabolized into acetyl-CoA for yielding citrate which is the source of producing NADPH [67]. So far, researchers have only made partial breakthroughs in understanding the glucose metabolic amplification, more efforts are still needed.

In addition, weak electrical activity [46] and a slight increase in $[Ca^{2+}]_i$ [54] were found when the β cells were treated with high glucose after the depolarization by high K^+ in the absence of diazoxide. It indicates that the amplifying pathway can not entirely explain this situation of glucose-induced insulin secretion, other factors should also be considered [43].

Mechano-sensing pathways

Besides the triggering and amplifying pathways, accumulating evidence suggests that additional ionic regulation coupled to glucose metabolism could mediate β-cell depolarization [68]. For instance, in isolated rat pancreatic β-cells, the cell volume was increased by 12% and 10% in response to 20 mM and 12 mM glucose, respectively, and this effect can be sustained when exposed to hexose, while treatment of non-metabolized 3-O-methylglucose was of no significant effect on cell volume change [69]. Furthermore, the glucose-stimulated cell volume increase showed a comparable influence on electrical activity induction [69]. Exposure to hypotonic solutions also induces β-cell swelling, transient electrical activity, and insulin secretion, which mimics the stimulatory effects of glucose to some extent, and these stimulatory actions can be inhibited by the anion channel blocker 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) [70, 71]. These findings indicate that the volume-regulated anion channel (VRAC) activation, possibly Cl^- efflux, contributes to glucose-induced depolarization in β-cells and insulin release [70-74]. A recent study unmasks a cell-swelling induced pathway involving the leucine-rich repeat (LRR) containing protein (Swell1) as a glucose sensor that mediates a swelling-induced chloride current and the β-cell membrane

depolarization that activates voltage-gated calcium channel (VGCC)-dependent calcium signalling and insulin exocytosis [75].

However, hypotonicity-induced insulin release persists even with the chloride channel blockers, DIDS or niflumic acid [76, 77]. Exposure to the hypotonic solutions leads to membrane depolarization and produces outwardly rectifying cation currents. Both these responses and hypotonic-stimulated insulin secretion can be suppressed by the cation channel blocker (Gd^{3+}) in isolated rat islets [78]. Thus, the proposition is that stretch-activated cation channels might be involved in the swelling-induced insulin secretion [78].

TRP (Transient Receptor Potential) channels belong to the mechanosensitive superfamily. An increasing number of data point to abundant expression of TRP channels in pancreatic β-cells and their potential regulation of insulin release. Expression of Trpc1, Trpv2, Trpv4, Trpm2-5 in mouse islets and Trpc1, Trpc4, Trpv5, and Trpm2 in rat islets or β-cells have been reported [79]. TRPV5-6 are found in human pancreas, and the transcripts of TRPM2, TRPM4-5 are detected in human islets [80, 81]. They are activated by a variety of stimuli including cell swelling, voltage, ligand binding, temperature, etc [82]. Trpm5 is involved in the regulation of Ca^{2+} oscillations and contributes to insulin secretion in pancreatic β-cells [83, 84]. *Trpm5* deficient pancreatic islets show reduced membrane potential, cytosolic free Ca^{2+} concentration and significant impairment in GSIS [84]. By measuring total glycosylated hemoglobin (TGH) from 997 pregnant women, mutations in TRPM6 are associated with higher TGH and leading to gestational diabetes mellitus [85]. Glucose and GLP-1 activated Trpm2 effectively depolarizes the cell membrane and initiates insulin secretion, whereas it is attenuated in *Trpm2* deleted mice [86]. Repeated observations made found that Trpv1 does not contribute to GSIS but might be involved in insulin sensitivity [87, 88]. Trpv2 channel is confirmed to be activated by osmotic-cell-swelling in mouse β-cells, resulting in membrane depolarization and subsequently voltage-gated Ca^{2+} channels activation and insulin secretion [89]. Recently, the mechanosensitive channels, Piezo1 (Fam38a) and Piezo2 (Fam38b) were identified as the long-sought-after mechanosensitive cation channels involved in mechanotransduction processes [90]. Their functions have since started to unravel, so are they potential candidates as sensors of cell swelling resulting from glucose metabolism and regulation of insulin secretion? This is the main question we will address in this thesis.

Ion channels

Ion channels are macromolecular complexes that span across the lipid bilayer of the cell membrane [91]. Different types of ion channels respond to either electrical activity (voltage-dependent ion channels), mechanical forces (mechanosensitive ion

channels), or chemical stimuli (ligand-gated ion channels), etc, result in small conformational changes to open the channels [92]. The deformation of the channels allows ions to enter or exit the cell. In general, the function of an ion channel is determined by the activity (conductance and open property) or the number of the channel in the cell surface [93].

As mentioned in the previous, Ca^{2+} is a mandatory signal and plays crucial roles in a variety of β-cell pathways involved in insulin secretion. β-cells possess numerous channels that influence Ca^{2+} signaling, such as voltage-gated Ca^{2+} channels and the newfound mechanosensitive ion channels. When the β-cells are exposed to stressful conditions during the pathogenesis of T2D, it results in perturbations in ion channel expressions, activities or localizations, which consequently alters Ca^{2+} handling. The defect in Ca^{2+} signaling of diabetic β-cells impairs insulin secretion and aggravates hyperglycemia [94].

Mechanosensitive Ion Channel: Piezo1

Mechanotransduction, the conversion of mechanical forces from the environment into biological signals, is crucial for survival. For instance, senses of touch, respiration, hearing, bladder control, the circulatory system and blood pressure regulation, etc, are regulated by mechanosensitive ion channels (MS channels also known as stretch-gated ion channels) [95, 96]. The existence of MS channels was first identified in 1984 in chick pectoral muscle [97]. Since then, MS channels have been found to be ubiquitously expressed in organisms from the three kingdoms of life including bacteria, archaea, and eukarya. Their structure and functions have been understood greatly, especially the discovery and cloning of Piezo1 and Piezo2 channels in 2010 [90] opened up the floodgates for a dramatic number of mechanotransduction-related research. Piezos are pore-forming homo-oligomer ion channels that can be stimulated by mechanical stimuli including membrane perturbation and osmotic imbalance, independent from the assistance of other cellular components [98, 99].

Piezo1 is a very large protein (see Figure 2) with a full-length of 2547 amino acids forming a trimeric propeller-like (some reported as bowl-like shape [100]) structure with three distal blades and a central cap [101]. Residues 1-2190 sense the mechanical forces and determine the open property of the pore of the channel laid in the C-terminal (residues 2189-2547) [99], which is responsible for the entry of positively charged ions with a slight preference for Ca^{2+} into the cells, and generates an overall depolarizing effect [90, 102]. To determine whether changed membrane tension is enough to activate Piezo1, overexpression of Piezo1 in the artificial cell membrane (cytoskeleton free) has been shown to directly sense the force from the bilayer tension [103]. The deformation of Piezo1 into a planar structure in response to membrane-perturbations-generated lateral membrane tension is responsible for

channel gating, as demonstrated by cryo-electron microscopy and high-speed atomic force microscopy [100, 104].

Piezo1 is broadly expressed at high levels in skin, bladder, kidney, lung and urothelium which are exposed to pressure and fluid flow [90, 105, 106]. General knockout of Piezo1 in mouse is embryonically lethal, owing at least in part to the disrupted development of the vasculature system [107, 108], indicating its essential role for fundamental life processes. Consistent with the phenotype, Piezo1 senses the extension of bladder [106], senses shear stress of blood flow for proper blood vessel development [107, 108], regulates red blood cell volume [109], controls cell migration and proliferation [110]. In humans, mutations of *Piezo1* resulting in altered channel functions have been linked to multiple hereditary human diseases, like dehydrated hereditary stomatocytosis (DHSt) which is linked to gain-of-function mutations in PIEZO1 ion channels [111]. *PIEZO1* (SNP rs9933309) was revealed as novel loci (within top 7 hits) harboring common variants associated with HbA1c in East Asians, affecting erythrocyte parameters rather than glucose metabolism, such variants could be relevant to the use of HbA1c for diagnosing diabetes [112]. Furthermore, the Piezo1 agonist yoda1 treatment induces insulin secretion in insulin-secreting β-cell lines and rodent pancreatic islets [113]. Taken together, these suggest that Piezo1 could be an important player for the regulation of insulin secretion in β-cells and pathogenesis of T2D, which are well studied in Paper I and II.

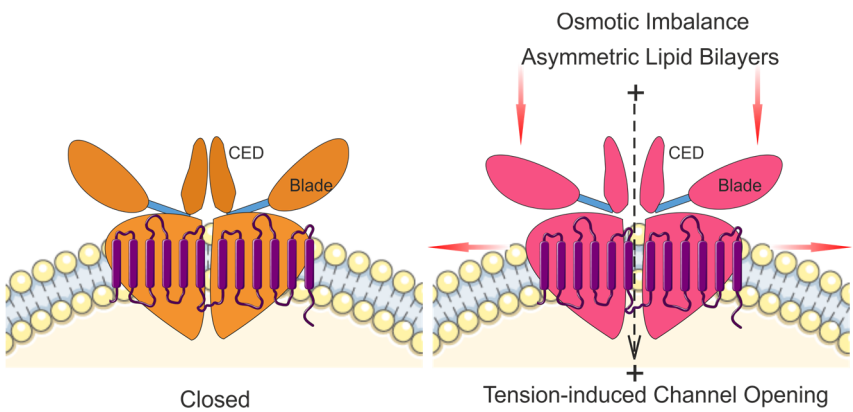

Figure 2 Illustration of the mechanotransduction and pore modules of the mPiezo1 channel [99-101, 114]. Changes of membrane tension driven by osmotic imbalance or asymmetric lipid bilayers are sensed by the transmembrane modules and the Piezo1 channel is open for positive ion entry. CED: C-terminal extracellular domain; Blade: Extracellular peripheral regions.

Voltage-Dependent Calcium Channels

Voltage-dependent Ca^{2+} channels (VDCC) take the most important role for the finely tuned balance of Ca^{2+} entry and efflux at the plasma membrane [82]. By doing

so, hormone secretion is tightly controlled to ensure proper pancreatic β-cell function.

Based on the structure, functional VDCCs contain the pore-forming α1 subunits which are subdivided into three main groups (see classification in Figure 3): the Cav1, Cav2, and Cav3 channels. The Cav1.1, Cav1.2, Cav1.3 and Cav1.4 channels encoded by *CACNA1S*, *-C*, *-D* and *F*, also known as L-type calcium channels, are sensitive to dihydropyridines (DHPs), such as isradipine [115, 116]. The Cav2.1 (also referred to as P/Q-type), Cav2.2 (N-type), Cav2.3 (R-type) channels are encoded by *CACNA1A*, *-B* and *-E*, respectively [117-119]. Both Cav1 and Cav2 channels are gated by high-voltage, termed as high-voltage activated channels (HVA). They are slowly inactivated during a sustained depolarization, so-called long-lasting activation [82, 120]. Cav3.1-3.3 channels, encoded by *CACNA1G*, *-H* and *-I* [121-123] are activated at a relatively lower voltage (∼−55 mV) and inactivated at ∼−40 mV which sustains in a brief depolarization [82].

The L-type calcium channels (mainly Cav1.2 and Cav1.3) are expressed in pancreatic β-cells and carry the majority of voltage-gated Ca^{2+} currents, influence GSIS [82, 120, 124, 125]. Cav1.1 and Cav1.4 are mainly found in skeletal muscle and retina cells, respectively, whereas scarcely detected in β-cells [125]. β-cell specific Cav1.2 knockout mice showed a ~45% decrease in the whole-cell Ca^{2+} current and abolished the first-phase insulin secretion resulting in glucose intolerance [120]. Silencing of *Cacna1d* (Cav1.3) decreases GSIS in insulin-secreting β-cells (INS-1 832/13 cells) and also impairs exocytosis in human islets [126]. Taken together, defects in the L-type calcium channels especially Cav1.2 and Cav1.3 are suggested to be involved in the development of diabetes.

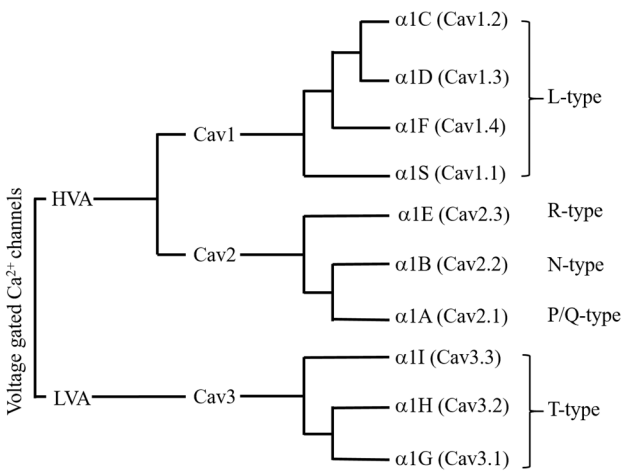

Figure 3 Classification of Voltage-dependent Ca^{2+} channels, modified from [127].

VDCCs also consist of multiple auxiliary subunits including α2δ, β and γ subunits attaching to the pore-forming α1 subunit and modulate the VDCC's functions. Either the Cav1 or Cav2 subtypes of VDCCs are capable to form a heteromeric complex, assembling with one of the β subunits (*CACNB1-4*) and one of the α2δ subunits (*CACNA2D1-4*); For Cav3 channels, these can be formed by α1 subunit alone without auxiliary subunits [128]. γ subunits contain 8 isoforms (γ1-8), γ4, γ6, γ7, and γ8 subunits are demonstrated to physically associate with the Cav1.2 channel in cardiac tissue [129, 130]. The γ1 subunit interacts with Cav1.1 channel in rabbit skeletal muscle [131]. Neuronal Ca^{2+} channels (Cav2.1 and Cav2.2) physically bind to γ2, γ3 and γ4 [132, 133]. Therefore, the α1 subunits of VDCCs except Cav3 channels also associate with α2δ, β and γ subunits (Figure 4).

Cavα2δ subunit

The α2δ subunit is primarily identified in the skeletal muscle together with Cav1.1 [134-137] and its molecular cloning was accomplished in 1988 [138]. Subsequently, N-type calcium channel is found to tightly interact with α2δ subunit in rabbit brain [139]. The α2δ subunit is encoded by a single gene, but during post-translational modification, it is cleaved into a glycosylated α2 protein which hangs extracellularly and a δ subunit spanning the membrane, these two separate proteins are connected by a disulfide bond as a mature subunit [125].

The α2δ and β subunits control the trafficking of VDCCs to the plasma membrane (PM) and also affect the channels' biophysical properties [140]. They also serve as stimulators for the expression of different Cav1 or Cav2 channels, either in functional expression or absolute amount of proteins at the plasma membrane, thus cause an increase of Ca^{2+} current amplitude and changes in current kinetics [141-145]. Cav2.2 channel expression in the plasma membrane is increased with α2δ-1 [146], and the resultant Ca^{2+} currents carried by Cav2.2 are induced by approximately 10-fold [147], indicating the importance of α2δ-1 on Ca^{2+} current density. Moreover, male mice with genetic ablation of α2δ-1 show a decreased Ca^{2+} influx through all types of functional VDCCs in pancreatic β-cells, which lead to the reduction of insulin secretion and glucose tolerance impairment [148]. However, the detailed cellular mechanisms regarding the single β-cell level needs to be explored (Paper III).

Cav γ4 subunit

The eight isoforms of the γ subunits are clustered into three subgroups: I. γ1, γ6, II. γ5, γ7, and III.γ2, γ3, γ4, γ8 according to the sequence homology and chromosomal linkage [149, 150]. Both γ1 and γ6 structurally lack a PSD-95/DLG/ZO-1 (PDZ)-binding motif and might also share physiological functions that distinct from most other γ subunits [150]. The pairwise amino acid identity of γ5 is closest to γ7 [149]. Cavγ2, γ3, γ4, and γ8 are regarded as transmembrane AMPA receptor regulatory proteins (TARPs) [151]. γ4 is broadly expressed in brain especially in fetal brain,

substantially distributes in lung and prostate, and is expressed relatively lower in pancreas, stomach, testes, etc [152]. RNA-sequencing data shows that *CACNG4* (γ4) is expressed in human β cell lines (EndoC-βH1 and -βH2 cells) [153]. Furthermore, calcium channels including *Cacna1d* (Cav1.3), *Cacna2d1* (Cavα2δ1), and *Cacng4* (Cavγ4) are downregulated in Goto-Kakizaki (GK) type 2 diabetic rats, which provides the molecular basis of the correlation between reduced L-type Ca^{2+} currents and low heart rate in GK rats [154]. The expression of γ4 in fetal brain shows a precise time correlation with the onset of neuronal differentiation, which indicates the potential role of γ4 in neuronal development, and γ4 might mediate cell differentiation by regulation of cytosol Ca^{2+} levels through VDCCs [155]. Several reports have shown differential modulations of γ4 on Ca^{2+} channel functions, it significantly shifts the Ca^{2+} current inactivation curves to more positive voltages when coexpressed with Cav3.1 [152]. When γ2 and γ4 subunits are coexpressed with Cav2.1, they shift the steady-state inactivation curve to more hyperpolarized potentials [156]. These data demonstrate that the γ4 subunit is part of the regulation of activation and inactivation of VDCCs. Collectively, these suggest to us to explore the potential roles of γ4 in healthy and diabetic conditions, which is developed in Paper IV.

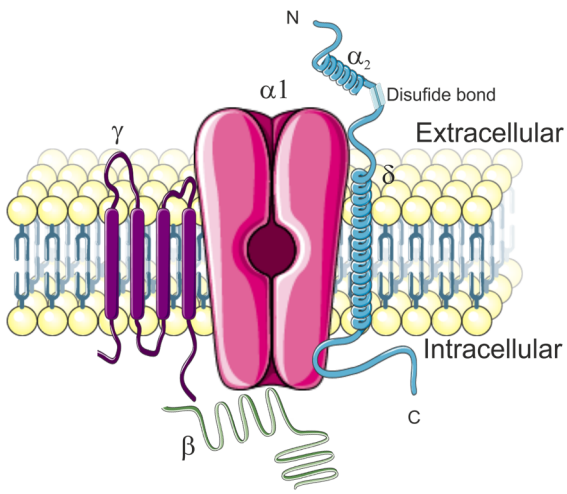

Figure 4 Structure of voltage-dependent calcium channel including α1, α2δ, β and γ subunits anchoring in the plasma membrane.

SNARE Proteins

Insulin exocytosis requires membrane fusion of insulin-containing granules mediated by a family of proteins referred to as soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. The docking, tethering and fusion of the insulin granules with the plasma membrane are highly controlled to ensure proper secretion of insulin into the extracellular environment [157]. The SNARE complex consists of Syntaxin, Snap-25 in the plasma membrane and Vamp2 (Vesicle-Associated Membrane Protein) in the secretory granule membrane. The association of these proteins is orchestrated by Munc18, Munc13 and RIM (the active zone protein, which plays a leading role in vesicle docking) [158-160]. A stable α-helical ternary complex is formed after finishing recruiting all of these proteins and is prepared for membrane fusion [157]. The detailed molecular machinery is comprehensively described in [39, 42, 157, 161].

Insulin-secreting cells express a full complement of SNARE proteins which are similar to those involved in synaptic vesicle exocytotic machinery in the neuron [161]. Disturbance of these proteins results in impairment of exocytosis [157, 162, 163]. The deduction of the peak secretion of first-phase GSIS has been partially attributed to the reduced predocked secretory granules which is mediated by Munc18a/SNARE complex [164, 165]. For instance, Munc18a has been demonstrated to control the first-phase insulin secretion [166] because of its key role in the priming of insulin vesicle for exocytosis [167]. Stx1a expression is found severely reduced in the islets of T2D, a β-cell specific *Stx1a* knockout mouse model shows decreased blood insulin level corresponding to the elevation of blood glucose, molecularly attributes to the deficiency of *Stx1a* remarkably decreases readily releasable pool and granule pool refilling, thus results in the impairment of both phases of GSIS [168]. SNAP25 expression is also decreased in the islets of T2D [165, 169] and is negatively correlated to HbA1c levels *in vivo*, positively correlated with GSIS *in vitro* [169], which suggests that SNAP25 is essential for insulin secretion in both human and mouse islets. VAMP/Vamp2 is highly expressed in both human and mouse β-cells [42] and it shows a negative correlation with HbA1c in human islets [169]. Vamp2 mediates the exocytosis of predocked insulin granules while Vamp8 is the major determining factor for the fusion of newcomer insulin granules [161]. Some novel ideas concerning the regulation of Munc18a/SNARE proteins are demonstrated in Paper III of this thesis.

Transcriptional factors

Mature pancreatic β-cells initiate from embryonic stem cells via an orchestrated cellular process known as differentiation. Differentiation is tightly controlled and coordinated by specific gene regulators in a time-dependent manner, and develops particular morphological and functional cellular features. For instance, maturation of β-cells enables them to release an appropriate amount of insulin in response to fluctuating glucose concentrations [170]. However, mature β-cells can lose their cellular identities and differentiated phenotypes to various degrees and regress to an immature or a precursor-like status under certain conditions, this process is termed as dedifferentiation which contributes to the loss of functional β-cell mass in T2D [6, 171-173]. In the progress of differentiation, numerous transcriptional factors (TF) are critically involved and play integral roles to direct cell destinies by regulating the transcription of their downstream genes in the line of cell maturation [174]. Examples are listed below as well as a summary of TFs in differentiation in Figure 5.

MafA and B

Among transcriptional factors (TF), MafA and B (musculoaponeurotic fibrosarcoma oncogene family A and B) appear to be islet-enriched TFs, play a fundamental role in the development of β-cell identity and functionality [175]. Expression of MafB is higher in rodent embryonic β-cells and is downregulated rapidly after birth, is substituted progressively by MafA in the progress of β-cells maturation, it is then restricted to α-cells 3 weeks after birth [176, 177]. Ablation of *MafB* in embryos reduces the amount of insulin$^+$ and glucagon$^+$ cells during the development without changing the total amount of endocrine cells, and the expression of MafA is delayed as well as the production of insulin$^+$ cells [177]. MafA is particularly expressed in mature β-cells, known as a maturation marker, which directly regulates insulin production and Glut2 [175]. Knockout of *MafA* in mice severely impairs glucose-, KCl-, or arginine-stimulated insulin secretion, leads to the development of glucose intolerance and T2D [178]. MafA expression is reduced in the diabetic mouse model (*db/db* mice) and human T2D islets, which suggests a potential signature of β-cell dysfunction [179]. Overexpression of MafA in immature rat islets and other insulin-secreting cells stimulates GSIS, which might owe to its regulation of a number of genes related to insulin secretion [178, 180, 181]. A dramatic amount of data, not limited to the above, provide support for the importance of MafA and B in β-cell development, regulation of insulin and other crucial genes [182-184].

PDX1

PDX1 (Pancreatic duodenal homeobox 1), also known as insulin promoter factor 1 (IPF1) manifests its role throughout all stages of pancreatic development [174]. Pdx1 can be detected in the early developing embryo from E8.5 in mouse [185] and week 4 gestation in human [186]. The developing of pancreas is arrested when Pdx1 is blocked from E11.5 in the pregnant mice, no β- or acinar-cells are found in the pancreas at birth [187]. Pdx1 binds to the insulin promoter, therefore, deletion of *Pdx1* in mature β-cells reduces insulin production and impairs glucose homeostasis [187]. β-cell specific knockout of *Pdx1* leads to severe hyperglycemia and the *Pdx1*-deleted cells rapidly achieve α-cell-like ultrastructural and physiological characteristics, and MafB starts to express in the reprogrammed cells, indicating Pdx1 as a crucial regulator of β-cell fate and is essential to maintain β-cell identity [188].

Ngn3

Neurogenin 3 (Ngn3) is also one of the most important TFs for endocrine development. *Ngn3* null mice are lack of islets, develop into T1D and die within 3 days after birth [189]. Ngn3 interacts with a few downstream TFs including Nkx6.1, Nkx2.2, Isl1, Pax4, Pax6, Pdx1 and NeuroD1, required for endocrine development and maintenance of cell identity [190-192].

In summary, TFs exert vital roles in the maturation process of pancreatic cells. Understanding the details of the mechanisms is a benefit for preventing the loss of maturity or restoring the differentiated state of β-cells in T2D.

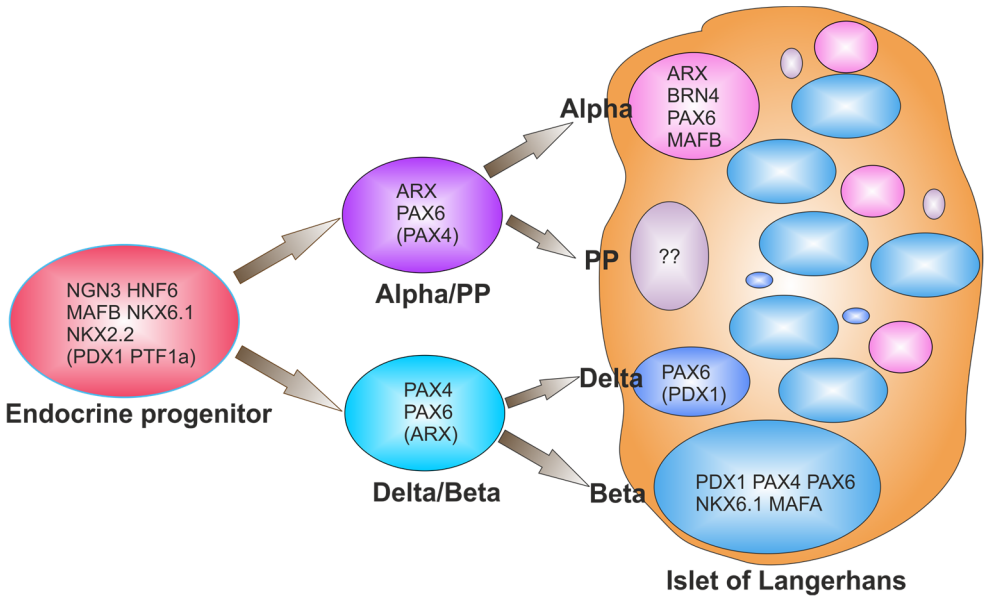

Figure 5 Selection of transcription factors (TFs) landmarks during pancreatic cell development.
Low expressions of TFs are indicated in parentheses. Modified from [193].

TCF7L2

A common genetic variant encoding the transcription factor 7-like 2 (TCF7L2, also known as TCF4) attributed to the single-nucleotide polymorphism (SNP rs7903146) has the strongest genetic risk for the development of T2D, revealed by genome-wide associate studies [194-196]. The risk T-allele of rs7903146 increases 1.5-fold of T2D risk and 2.4-fold in heterozygous and homozygous carriers, respectively, corresponding to a 21% population risk [197]. From a cellular functional view, TCF7L2 is a key effector in the Wnt signaling pathway which is involved in cellular growth and organogenesis [196, 198, 199], as well as in adipogenesis [195], β-cell survival and functions in human and mouse islets [200]. A TCF7L2-regulated transcriptional gene network, affecting insulin production and processing in human and rodent pancreatic islets, has been identified by RNA-sequencing. Among these genes, *ISL1*, *MAFA*, *PDX1*, *NKX6.1*, *PCSK1*, *2* and *SLC30A8* are highlighted and confirmed to be associated with *TCF7L2* [14]. TCF7L2 also influences insulin secretion by regulating the transcription of various proteins such as proglucagon and glucagon-like peptides (GLP-1 and 2) [201, 202]. Silencing of *Tcf7l2* markedly reduces the mRNA expression of *Cacna2d1* (the aforementioned Cav channel subunit) but does not influence the genes controlling Ca^{2+} signaling and exocytosis [14]. Therefore, it is worthy to verify the regulatory effects of Tcf7l2 on Cavα2δ1 as well as how this influences Ca^{2+} signaling and insulin secretion in pancreatic β-cells, which is detailed in Paper III.

Aims

Development of diabetes, especially T2D, attributes not only to genetic factors, but also environmental input. As aforementioned, insulin-secreting pancreatic β-cells can adjust their state in response to high glucose or osmolarity imbalance versus the extracellular space. Increasing evidence has indicated the involvement of mechanosensitive channels in this respect. However, the effectors behind the regulatory pathway are not fully unraveled. The transcription factor TCF7L2 is the strongest diabetes risk gene, MafA is important for the development and identity maintenance of β-cells. VGCCs play key roles in insulin secretion and highly correlate with T2D. However, the regulation of TCF7L2 and MAFA on the auxiliary subunits such as α2δ, γ subunits associated with Cavα1 are not completely understood in islet β-cells. In this thesis, we aim to develop a novel insulin secretion pathway involving mechanosensor Piezo1 *in vitro*/ *in vivo*, also the roles of Tcf7l2 and MafA in controlling β-cell function via the auxiliary subunits α2δ1, γ4 subunits, respectively.

The Specific Aims of the Thesis:

I. To explore the involvement of the mechanosensitive channel Piezo1 in the insulin secretion pathway in pancreatic β-cells.

II. To investigate the role of Piezo1 *in vivo* in β-cell specific *Piezo1* knockout mice.

III. To study the regulation by the diabetes risk gene Tcf7l2 of the voltage-gated calcium ion channel subunit Cavα2δ-1 and, in turn, Ca^{2+} signaling and insulin secretion.

IV. To examine the physiological mechanisms whereby MafA regulates Cavγ4 affects pancreatic β-cell function.

Material and Methods

Here only the methods that needed more detailed descriptions will be considered. Readers are referred to the original papers for other methods used in the studies included in this thesis.

Generation of β-cell specific *Piezo1* knockout mice

The Cre-loxP recombinase system has been commonly utilized to delete genes or activate reporters in pancreatic cells in mice. It is an indispensable tool to investigate the cell-, tissue- and/or developmental stage-specific functions of the target gene in the pathophysiology of diabetes. The P1 bacteriophage-derived Cre recombinase is a 38 kD homotetramer, recognizes the 34 bp loxP sequence and excises the loxP-flanked DNA sequence (normally contains one or more exons). The Cre-mediated recombination is guided by the orientation of loxP sites. Inversion or excision occurs when the loxP sites localize on the same strand of DNA, while it performs insertion when they are on separate strands [203]. Similarly, to activate the expression of the target gene conditionally, the Cre recombinase recognizes the allele containing a lox-stop-lox (LSL) sequence to induce the expression of the coding sequences [204]. Recombination mediated by Cre can be controlled by regulating the timing or spatial distribution of Cre expression [203], like the line Cre^{ER} which enables temporal regulation of Cre recombination by activation of tamoxifen [205]. There are at least 79 pancreas-specific Cre driver lines which can be subdivided into four categories according to the Cre expressed cell types: endocrine, exocrine, ductal and pancreatic progenitor cells [204]. The first three categories are distinguished by cell-type-specific genes such as hormones or digestive enzymes that mark individual cells in the pancreas; The fourth category is normally used for studies of development and functions of the pancreas. The new floxed alleles development by introducing the embryonic stem cells with mutant allele into the germline of mice has been improved which allows a diverse generation of conditional knockout mice.

Global knockout of *Piezo1* mouse model is embryonically lethal [107, 108], to evaluate the specific function of Piezo1 in pancreatic β-cells *in vivo*, β-cell-specific *Piezo1* knockout (KO) mice are therefore warranted. To this end, we use the mice expressing rat insulin 2 gene promoter-driven Cre (RIP-Cre^{+}) [206] and the floxed

Piezo1$^{tm2.1Apat/J}$ (P1^{f}) mice whose Piezo1 has been engineered to incorporate loxP sites from exon 20 to 23 (Stock #029213, The Jackson Laboratory) [109] in this thesis. The two lines of mice were mated to obtain RIP-Cre^{+}. P1$^{f/+}$ mice, which were then crossed with P1^{f} to get RIP-Cre^{+}.P1$^{f/f}$ KO mice (Figure 6). The tail samples from the litters were genotyped following the protocol from the Jackson Laboratory, the gene depletion state was further confirmed by testing Piezo1 mRNA and protein levels in whole islets and single β-cells, respectively.

Due to the limitations of Cre/loxP system and the known deficiencies of a given mouse line, one has to prudently draw scientific conclusions from the results by using this strategy. The RIP-Cre line (Tg(Ins2-cre)^{23Herr}) we used was confirmed normoglycemic and the islets from these mice are histologically normal as well [206], the blood glucose post intraperitoneal glucose tolerance test (IPGTT) of Cre and floxed *Piezo1* mouse line was also compared by us and both appear normal, but the leaky expression in the neuroendocrine cells and the brain should be also kept in mind [207, 208].

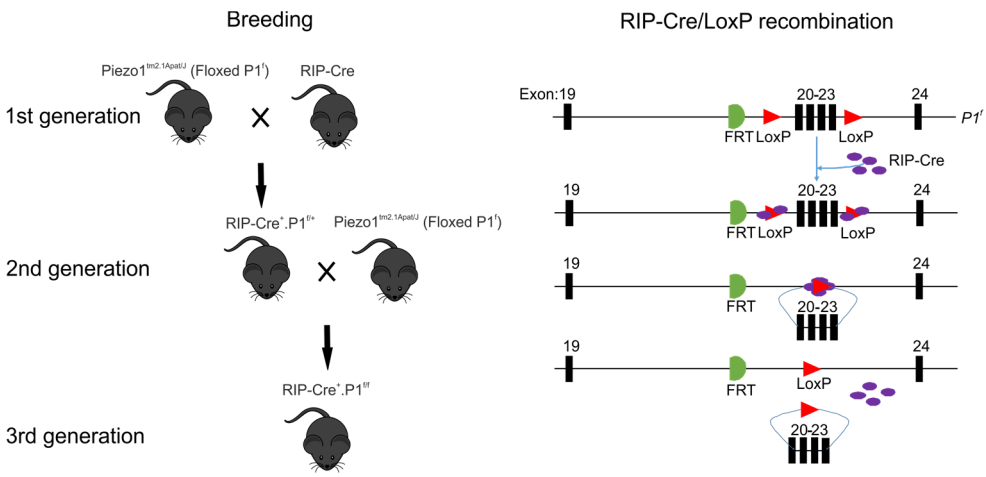

Figure 6. Scheme illustration of generation of β-cell specific *Piezo1* knockout mice. Generation of Piezo1$^{tm2.1Apat/J}$ (P1^{f}) refers to [109], FRT: FLP recombinase target

Pancreas perfusion *in situ*

To investigate the physiological functions from both endocrine and exocrine tissue in the pancreas, *in situ* pancreas perfusion is performed [209, 210]. In contrast to the studies in isolated islets, this method mimics the *in vivo* conditions meanwhile eliminates the secondary effects of other organs. Pancreas perifusion has the advantage of detecting small changes in a dynamic view of insulin secretion in response to different pharmacological drugs and/or nutrients (e.g. glucose). This

method has been utilized for investigating physiological pancreatic functions related to T2D [211-214]. It is a useful tool for the exploration of potential therapeutic candidates especially to insulin secretion regulation [215].

First, the anticoagulant heparin (2000 units/kg) is intraperitoneally injected in the non-fasted C57BL/6J mouse to prevent the blood clots from compromising the system. Then the mouse is sacrificed by a rising concentration of CO_2. After opening the abdominal cavity, the renal, hepatic, splenic, superior mesenteric and inferior mesenteric arteries are ligated, the aorta is tied off above the level of the pancreatic artery (A double ligature is preferable to prevent the leakage during the perfusion due to incomplete ligation). A silicone catheter connected cannula Butterfly needle (27 G) is placed in the celiac aorta which is the site for the entry of testing solutions. The perfusate is collected at an interval of 1 min via the portal vein with a silicone catheter connected cannula Butterfly needle. The mouse is kept on a heating pad (37°C) during the perfusion. The pancreas is perfused with a mixture of Krebs-Ringer buffer containing 1mg/ml BSA and glucose/drugs as indicated (filtered with Filtropur S 0.2 unit) at a rate of 1 ml/min using a KDS Legato 100 series syringe pump. The buffer is priorly equilibrated with O_2/CO_2 (95:5) resulting in a pH range of 7.28 to 7.40. Preperfusion with 2.8 mM glucose Krebs buffer is required to flush out the blood as well as to maintain a basal level of insulin secretion and pancreas function. The production of insulin in the collected perfusate is a reflection of the responsiveness to glucose/drugs/hypotonicity/other secretagogues and is measured by radioimmunoassay (RIA).

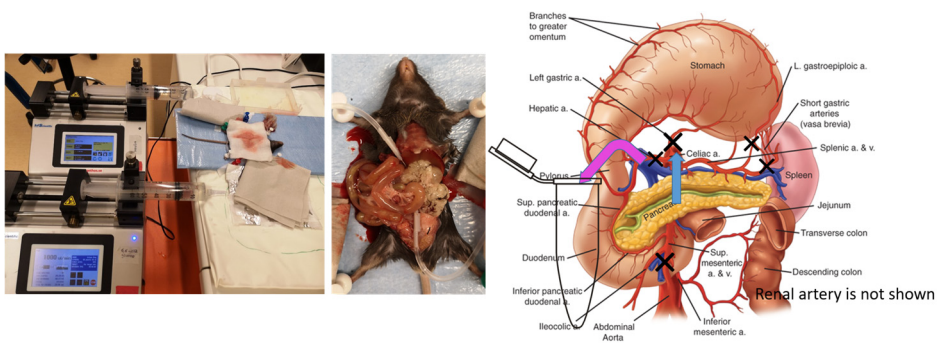

Figure 7. The perfusion setup (left) and illustration of ligations in arteries (right).

Patch clamp and capacitance measurement

The patch-clamp technique was developed in 1976 by Neher and Sakmann, it has been widely used to record the whole-cell or single-channel currents through the ion channels embedded in the cell membrane [216, 217]. This technique is commonly

applied to study the electrophysiology of specific ion channels in excitable cells such as neurons and pancreatic β-cells.

By controlling the voltage (voltage clamp) or current (current clamp), the experimenter can record the resulting changes in current or voltage (membrane potential) across the cell membrane, respectively. Depending on the specific purpose of the study, several variations can be selected, including the whole-cell patch and perforated patch which allow investigators to study the summed electrical activities of the ion channels in the entire cell, and the inside-out/outside-out techniques in which a section of membrane is removed from the cell to study the behavior of single ion channel in the excised patch.

In this thesis, we used voltage clamp to investigate the Ca^{2+} currents in a whole-cell configuration. In practice, an AgCl coated silver electrode is placed into the micropipette (thin and blunt-tipped) filled with intracellular solution, the pipette is pressed onto the cell surface, the experimenter applies gentle suction to form a giga-seal with high electrical resistance (>1 GΩ). As soon as the giga-seal is established, voltage is simultaneously applied, the patch of membrane is then ruptured by a pulse of negative pressure (short suction) in the whole-cell configuration. The electrode in the micropipette now is a part of the electric circuit. It records real-time results about both current (magnitude and direction of the ion flow) and the time for activation or inactivation of the individual channels. Here, the Ca^{2+} currents were monitored using a software (Pulse or Patchmaster) controlled amplifier (EPC9 or EPC10, HEKA) connected to the electrode.

In patch-clamp, a good and stable gigaseal between the pipette and cell membrane is the fundamental requirement for achieving stable configurations and avoiding current leaking. For whole-cell configuration, the cytosolic content in the cell is replaced over time by the intracellular solution in the pipette. The composition of the solution can be adjusted to fit the purpose of the study, however, it might also affect certain cellular functions by dialyzing the interior of the cell. Therefore, perforated patch is an alternative method to evade this issue. The membrane of the cell on detection is perforated by pore-forming antibiotics (e.g. amphotericin), the cell is maintained as integrated which only allows a permeability of small monovalent ions (<200 Dalton). Therefore, this configuration is more stable than the conventional whole-cell mode and also prevents rundown of currents [216, 218, 219], while it's more demanding for a stable setup of the recording system and also time-consuming.

Patch clamp is not only used for recording the activities of ion channels, granule exocytosis can also be measured. The cell membrane serves as an electrical capacitor due to its lipid bilayer structure. The capacitance (C) is calculated according to equation [220] as below:

$$C = (\varepsilon \times A)/d$$

Where A represents the area of the cell surface, ε and d are constant which stand for the specific capacitance (0.9 fF/μm^2), and the distance between the bilayer of phospholipids, respectively. Therefore, the changes of capacitance (C) proportionally reflect changes in cell surface area (A).

In β-cells, exocytosis occurs when insulin granule fuses with the cell membrane and it leads to an expansion of the cell surface area. Hence, this increase in cell surface can be detected as an increase in capacitance representing exocytosis [221]. The fusion of a single vesicle is estimated to produce an increase in membrane capacitance of 3.6 fF [39]. To note, this method is not able to distinguish the exocytosis and concomitant endocytosis since it records the total changes in cell surface area. However, the maximum rate of endocytosis is much lower than that of exocytosis [222]. Moreover, upon stimulation, fusion of synaptic-like vesicles or organelles also occurs which might affect the results [39], even though it only contributes ~1% to the total capacitance [223]. These should be kept in mind during data interpretation.

In this thesis, INS-1 832/13 cells, dispersed human or rodent islets were used for the experiments. The pipettes had an average of resistance at ≈5.5 MΩ and the temperature in the bath solution was maintained at 32°C. Holding- and test-pulse were conducted by the software-controlled amplifier with a specific protocol to record the Ca^{2+} currents. The increase in membrane capacitance was evoked by a train of ten membrane pulses from -70 mV to 0 mV for 500 ms applied at 1 kHz sine wave. The first two depolarizations indicate the first phase of insulin release due to the exocytosis of docked and primed readily releasable granules (RRP), and the next eight depolarizations represent the second phase of insulin secretion from the reserve pool [39]. To identify the pancreatic β-cells in rodent, inactivation properties of Na^+ channel were detected. The half-maximal inactivation of Na^+ channels in β-cells is at ~78mV, whereas it's at ~40 mV and ~20 mV for α-cells and δ-cells, respectively [224]. However, this method does not apply to human islet cells, instead, cells bigger than 9 pF are considered to be β-cells [225].

Results and Discussion

Paper I and II

Upregulated Piezo1 expression in T2D islets

RNA-sequencing (RNA-seq) data verifies the presence of PIEZO1 in human tissues including pancreatic islets and a similar expression pattern is also observed in a panel of mouse tissues by qPCR. More importantly, PIEZO1 expression is significantly higher in islets from T2D and diabetic db/db mice. A similar enhanced expression pattern of Piezo1 is also found in ageing transgenic Alzheimer's rats [226] and prostate cancer cell lines/tumor tissues [227]. Mechanical activation of Piezo1 results in development of pancreatitis [228] and exerts an important role in cardiac remodeling [229]. These suggest that upregulation of Piezo1 might be a risk/causative factor for T2D.

Immunostaining further confirms that expression of Piezo1 in single α- and β-cells is comparable in both human and mouse islets, but interestingly with different localizations. Piezo1 is found in the cytosol and membrane area of β-cells, while in α-cells the nuclear expression of Piezo1 is much more prominent. Under standard culture conditions (10 mM glucose), α-cells exhibit more nuclear Piezo1 than the β-cells. This is probably related to the opposite physiological triggering of α- and β-cell activity. For instance, at high glucose, glucagon secretion is suppressed while insulin is activated.

Hyperglycemia induces translocation of Piezo1 into the nucleus

Since Piezo1 is present in both human and mouse β-cells, it prompted us to test whether elevated glucose affected its intracellular distribution. The data in dispersed/intact islet demonstrate that exposing β-cells to high glucose promotes the intracellular translocation of PIEZO1 from the cytosol and membrane into the nucleus.

The proportion of Piezo1 in the nucleus of islet β-cells in hyperglycemic/diabetic *db*/*db* mice (fed plasma glucose: >25 mM) is significantly higher than in β-cells from normoglycaemic control mice. Interestingly, this nuclear Piezo1 can be

relocated to the cytosol and membrane area after incubation in normal glucose. The intracellular distribution of PIEZO1 in β-cells from non-diabetic and T2D donors were also compared, but no significant difference was observed. Presumably, the diagnosed diabetic donors were well-treated with appropriate hypoglycemic medication, resulting in near-normoglycemia, which might affect the results. Ideally, islets from undiagnosed T2D donors with high HbA1c should be the critical comparable group versus healthy donors, to correctly assess the real distribution of Piezo1 under hyperglycemia. This suggests that Piezo1 distribution is glucose-dependent. As a mechanosensitive channel, a decreased amount of membrane Piezo1 could affect the proper function for ion passage, whereas the role of internalized Piezo1 is worthy of further exploration. Another piece of evidence from epithelial cells also shows that Piezo1 redistributes to the area close to the nucleus from the cytosol when cells are in dense regions [230]. Altogether, these findings demonstrate that localization of Piezo1 is under metabolic regulation and also raise interesting possibilities that β-cells respond to various environmental stimuli by translocation of Piezo1.

To determine which domain of Piezo1 controls the intracellular trafficking, mouse Piezo1 fragments from the pore-forming C-terminal (aa2189-2547) were overexpressed in INS-1 832/13 cells and the cells were challenged with different concentrations of glucose. We find that Piezo1 aa2458-2547-GFP (the inner helix of the pore) exhibits redistribution from nuclear to cytosol in response to high glucose while no translocation for Piezo1 aa2189-2547-GFP (comprising the entity of central pore) or aa2189-2458-GFP (corresponding to the outer helix of the pore). A similar phenomenon, that the nucleus localization of C-terminal (1592 to 2521) translocates to the cytosol and surface area, is also observed when co-expressed with the N-terminal (1-1591) of Piezo1 [231]. Taken together, the C-terminal inner helix part of Piezo1 is required for intracellular trafficking. However, we acknowledge that the exact sites responsible for sensing the metabolic state and translocation to the nucleus remain to be identified.

Piezo1 is important for swelling-induced insulin secretion

The passage of cations, including Ca^{2+}, through Piezo1 is associated with membrane depolarization [90, 107]. As expected, hypotonic swelling-induced Ca^{2+} signaling and membrane potential is inhibited by the Piezo antagonist GsMTx4 in INS-1 832/13 cells. Low expression of Piezo2 in INS-1 832/13 cells, also in mouse and human β-cells [22, 23], points to the predominant importance of Piezo1 in mechanosensory-induced depolarization of the β-cell membrane. In addition, GsMTx4 abolishes hypotonic swelling-stimulated insulin secretion, collectively indicating the involvement of Piezo1 in this respect.

Pancreatic islets are richly vascularized and the blood flow exhibits great variation *in vivo* [232]. Piezo1 has been reported to be activated by shear stress [13, 25, 26]. We mimicked this in experimental settings and shear stress indeed induces insulin secretion, but it is persisted by the application of GsMTx4. According to the activation mode of Piezo1 [99-101, 114] by membrane tension, hypotonicity-induced swelling and shear stress-driven forces provide different kinds of lateral friction for stimulating Piezo1, which might represent distinct pathways. Collectively, these data demonstrate that whereas β-cells respond to both shear stress and hypotonicity with stimulation of insulin secretion, only the latter effect reflects activation of Piezo1. Together with other reports, we conclude that β-cells are mechanosensitive [69, 75, 233]. These findings indicate a novel pathway involving Piezo1 regulating mechanical forces-induced insulin release. This appears to function independently of VRAC [70, 75, 234] which has been suggested to associate with hypotonicity/glucose-induced insulin secretion.

Piezo1 controls cytosolic Ca^{2+} homeostasis in β-cells

We next compared the effects of glucose and the non-metabolizable hexose mannitol (as an osmotic control) on $[Ca^{2+}]_i$. High glucose, but not mannitol, exerts a robust stimulatory effect of $[Ca^{2+}]_i$ and silencing of *Piezo1* decreases this metabolic $[Ca^{2+}]_i$ elevation. Importantly, the $[Ca^{2+}]_i$ evoked by high extracellular K^+ (70 mM) is unaffected by silencing *Piezo1*. The specific Piezo1 activator yoda1 [235] increases $[Ca^{2+}]_i$ when applied at 2.8 mM glucose in primary human and rat β-cells. In contrast, GsMTx4 abolishes glucose-induced $[Ca^{2+}]_i$ oscillations in both human and rat β-cells whilst not affecting the peak produced by high-$[K^+]_o$ depolarization. Furthermore, activation of Piezo1 by yoda1 depolarizes the β-cell membrane whereas silencing of *Piezo1* inhibited high glucose-induced depolarization. An abundance of reports has revealed the regulation of Piezo1 on Ca^{2+} homeostasis in insulin-secreting cell lines [113], urothelial cells [106], astrocytes [226], prostate cancer cell lines [227], cardiac fibroblasts [229], endothelial cells [236]. These support our findings that Piezo1 is particularly important for controlling Ca^{2+} signaling by sensing uptake and metabolism of glucose.

Shear stress has previously been reported to cause depolarization and activate VGCC in the adjacent vascular smooth muscle cells [26]. This suggests an additional effect on VGCC which is triggered by the mechanical stimuli-enhanced β-cell basal depolarization via Piezo1. RNA-seq and qPCR data demonstrate that *Piezo1* mRNA expression has either positive or negative correlations with Ca^{2+} channels. dSTORM super-resolution TIRF imaging reveals the physical association between PIEZO1 and Cav1.3. These primary data provides great information to verify our hypothesis but needs further confirmation. For example, which Ca^{2+} channel plays the predominant role in response to Piezo1-mediated depolarization requires careful validation.

Piezo1 is required for glucose-stimulated insulin secretion in β-cells

Next, the function of Piezo1 in insulin secretion was investigated. GsMTx4 abolishes glucose-stimulated insulin secretion in both human, rat islets and INS-1 832/13 cells. Pancreas perfusion also reveals the inhibitory effect of GsMTx4 on insulin secretion under quasi- physiological conditions. Silencing of *Piezo1* manifests a similar reduced effect on GSIS. However, *Piezo1* silencing does not affect high-K^+ induced insulin secretion which is in line with the $[Ca^{2+}]_i$ imaging data.

The activation of Piezo1 by yoda1 dramatically increases glucose-stimulated insulin release. The closure of K_{ATP} channel is central in the insulin triggering pathway [42, 237, 238]. To study whether Piezo1 exerts via a K_{ATP} channel-independent action, the K_{ATP} channel opener diazoxide (DZX) was used for testing. The stimulatory effect of yoda1 at basal is abolished by DZX, while at high glucose, yoda1 retains a minor stimulatory effect in the presence of DZX. These findings suggest that Piezo1-activated insulin secretion can occur independently from the triggering pathway but can be markedly enhanced by a series of actions after glucose metabolism (e.g. depolarization after the closure of K_{ATP} channel). As expected, either DZX, yoda1 or the combination of the two have no effect on high K^+-stimulated insulin secretion, which demonstrates their action on glucose sensing in the β-cell. Hence, inhibition of K_{ATP} channel by glucose metabolism is required for Piezo1-mediated GSIS.

We also tested the effect of yoda1 *ex vivo* by pancreas perfusion. 0.01% DMSO was used as the solvent for yoda1 and that interfered with insulin secretion in the perfused pancreas. In control experiments, glucose stimulated insulin secretion for <3-fold. However, the stimulatory effect of glucose was ~7-fold when the experiment was repeated in the continuous presence of yoda1. It is notable that the effect of yoda1 was restricted to the 1st phase (t=12-16 min) glucose-induced insulin secretion with no stimulation observed during the 2nd phase (t=25-40 min).

SWELL1 has been indicated to sense glucose-induced cell swelling and mediate insulin secretion [75]. Piezo1 seems to operate in parallel with Swell1 and both of them contribute to the swelling-induced signaling pathway, since either silencing these genes alone or double knockdown has similar effects on hypotonicity-induced $[Ca^{2+}]_i$ signaling. This indicates that there might be more than one system responding to the glucose-induced swelling and mediate insulin secretion.

Regulation of Piezo1 on global gene expression and hypotonicity-stimulated insulin secretion

The shift of Piezo1 into nuclei under hyperglycemia suggests that Piezo1 might also play roles in gene transcription. To this end, mRNA-sequencing unravels the genes

regulated by Piezo1. 3300 genes in total are significantly differentially expressed, among which 1452 genes are downregulated and 1394 genes are upregulated after silencing *Piezo1*. Gene Ontology (GO) terms enrichment show that 58 genes in "regulation of intracellular transport" and 42 genes in "nucleocytoplasmic transport" are downregulated by silencing of *Piezo1*. These provide interesting candidates for further study of the mechanism for Piezo1 redistribution under hyperglycemia in β-cells. More intriguingly, 68 genes involved in "positive regulation of secretion" are upregulated due to *Piezo1* silencing. Cocaine- and amphetamine-regulated transcript (CART) ranks in the top 1, the upregulation of mRNA expression of the top genes is verified by qPCR analysis.

To continue identifying the functions, *Piezo1* and/or *Cartpt* were silenced for measuring hypotonicity-stimulated insulin secretion (HSIS). Surprisingly, silencing of *Piezo1* dramatically increases HSIS which is opposite to the findings by Piezo1 channel blocker GsMTx4. This effect of increased HSIS can be counteracted by keeping the low expression of *Cartpt* by double knockdown of *Piezo1* and *Cartpt*.

Intriguingly, the K_{ATP} channel opener diazoxide (DZX) eliminated HSIS in either non-targeting siRNA treated cells (si-Ctrl) or *Piezo1*-silenced cells indicating the involvement of K_{ATP} channel-closure mediated membrane depolarization in HSIS.

Ca^{2+} imaging and membrane potential were performed to further demonstrate the mechanisms behind the increased HSIS after silencing of *Piezo1*. Hypotonicity-induced $[Ca^{2+}]_i$ is significantly reduced by silencing of *Piezo1* which is in line with previous data. In contrast, hypotonicity stimulated-membrane depolarization is enhanced which might explain the increased HSIS after silencing of *Piezo1*. *Piezo1* knockdown results in secondary changes in gene expression, especially *Cartpt*. This might explain the discrepancy between GsMTx4 (merely block Piezo1) and silencing of *Piezo1*. Taken together, these results unequivocally demonstrate that hypotonic swelling-induced insulin secretion requires K_{ATP} channel-closure mediated membrane depolarization and also indicate that Piezo1 possesses diverse functions other than as a mechanosensitive cation channel. The distinct effects of silencing *Piezo1* on GSIS and HSIS also suggest that HSIS follows different pathways from GSIS, for instance, Ca^{2+} is not a necessity for HSIS [239].

Glucose homeostasis in β-cell-specific *Piezo1* knockout mice

At this point we wanted to know the function of Piezo1 *in vivo*. *Piezo1*-deficient embryos die at midgestation due to defects in blood flow activated vascular development [108]. To this end, β-cell-specific *Piezo1* knockout mice were generated by using RIP-Cre mice and floxed *Piezo1* mice. The littermates were genotyped, confirmed by qPCR, and single islet cell immunostaining. Collectively, all results pointed to successful generation of β-cell-specific *Piezo1* knockout mice.

Then, glucose utilization *in vivo* was tested by intraperitoneal glucose tolerance test (IPGTT) at different ages in male and female Cre^+ (control), $Cre^+.P_1^{f/f}$ (homozygote Piezo1 knockout) mice without prior fasting. Male $Cre^+.P_1^{f/f}$ mice at 5-8 weeks show a higher blood glucose post-IPGTT than the control mice. There is no difference between these groups of mice at 15 weeks. More intriguingly, homozygote knockout of Piezo1 lowers the blood glucose when the mice are older than 25 weeks and the blood glucose post-IPGTT tends to return to the basal more rapidly in the $Cre^+.P_1^{f/f}$ mice. Deletion of Piezo1 in female does not affect the blood glucose concentration before 15 weeks of age, however, the blood glucose is markedly reduced compared to the Cre^+ mice above 25 weeks.

Insulin secretion in β-cell-specific *Piezo1* knockout mice

To explain the phenotypes above, static incubations of isolated islets from male Cre^+, $Cre^+.P_1^{f/f}$ mice for insulin secretion were performed at comparable ages as above. GSIS in young 7-8 weeks old male mice is impaired in $Cre^+.P_1^{f/f}$ mice. Both groups of 15-week old mice show a similar insulin-secreting capacity in response to high glucose. Interestingly, GSIS in the >22-week of age $Cre^+.P_1^{f/f}$ mice is remarkably increased. Taken together, these insulin secretion data perfectly echo the IPGTT results. *Piezo1* ablation in β-cells surprisingly appears to have an age-dependent effect *in vivo*. Knockout of *Piezo1* transiently impairs glucose tolerance and insulin secretion in young mice, which is in line with our data *in vitro*. In terms of blood glucose, control mice show slight glucose intolerance and a lowered insulin release upon glucose stimulation with increasing age, whereas lack of Piezo1 in β-cells in older mice results in better glucose utilization and an increased GSIS. These data demonstrate either bidirectional functions of Piezo1 at different ages or that other age-dependent factors compensate for *Piezo1* depletion in older mice. The RNA-seq data in *Piezo1*-silenced INS-1 832/13 cells might support the latter hyperthesis. For example, silencing of *Piezo1* results in the upregulation of mRNA expression of 68 genes involved in "positive regulation of secretion" and the amphetamine-regulated transcript (CART) is within the top 1. Reports show that CART is expressed in the majority of rat islet cell types (except ghrelin cells) within a period of two weeks after birth, CART expression later on is restricted to somatostatin cells [240]. Endogenous β-cell CART promotes both expression and secretion of insulin through the regulation of exocytotic machinery and key β-cell transcription factors [241]. Therefore, we hypothesize that β-cell-specific knockout of *Piezo1* might upregulate the expression of Cart in β-cells by age, this compensatory effect by Cart increases functional β-cell mass and long-term insulin secretion [242]. This hypothesis can be verified by testing the expression of those Piezo1-upregulated genes including Cart and β-cell proliferation in the knockout mice.

Deletion of the mechanosensitive channel Piezo1, as expected, reduces the peak response of hypotonic swelling-induced insulin secretion compared to control mice,

and also shows a tendency for decreased accumulated insulin secretion. The reduced hypotonicity-induced Ca^{2+} signaling after silencing of *Piezo1 in vitro* might provide an explanation, but it deserves further investigation.

Electrical activity and calcium homeostasis in β-cell-specific *Piezo1* knockout mice

The previous data point to silencing of *Piezo1* or activation of Piezo1 by yoda1 in INS-1 832/13 inhibits or induces glucose-induced membrane potential, respectively. To further study the possible changes in glucose-stimulated electrical activity after deletion of *Piezo1* in β-cells, the membrane potential in intact pancreatic islets was recorded during perifusion with increasing glucose concentrations from 5 mM to 16.7 mM. The islets were isolated from young mice (age 5-7 weeks): Large cells (> 8 pF) without Na^+ currents were categorized as β-cells. Strong depolarizing oscillations upon acute high glucose stimulation were observed in Cre^+ mouse islet β-cells, whereas the electrical activity in *Piezo1*-depleted pancreatic β-cells was dramatically reduced. However, membrane depolarization caused by the K_{ATP} channel inhibitor tolbutamide was less influenced by the ablation of Piezo1, which is in line with our previous data. These results demonstrate that Piezo1 is required for β-cell membrane depolarization, and also suggest that voltage-gated Ca^{2+} channel mediated Ca^{2+} currents are downstream effects of Piezo1 activation.

As expected, high glucose-stimulated Ca^{2+} concentrations from dispersed $Cre^+.P_1^{f/f}$ mouse (15 weeks old) islet are decreased compared to the control mice. Single β-cell under perfusion was selected for $[Ca^{2+}]_i$ analysis, demonstrating that Ca^{2+} signaling per se upon stimulation is reduced due to the deletion of Piezo1. Together with the data *in vitro*, these indicate the importance of Piezo1 in intracellular Ca^{2+} handling.

Surprisingly, β-cells from $Cre^+.P_1^{f/f}$ mice respond to yoda1 the same extent as the Cre^+ mice. This may be due to the complicated structure and size of the Piezo1 protein (51 exons). A frameshift in the *Piezo1* gene after deletion of exons 20-23, located in the mechanosensitive part of *Piezo1*, might lead to a folded protein product which has a similar structure to the C-terminal of *Piezo1* comprising the yoda1 binding site, as previously suggested [243]. An alternative explanation might be that yoda1 also activates Trpv4-dependent Ca^{2+} signaling [244], which requires further investigation to be resolved.

Highlights

1. Expression of the mechanosensitive channel Piezo1 is upregulated in T2D and shows heterogeneous localization in pancreatic α- and β-cells.
2. Cytosolic and membrane-localized Piezo1 in healthy pancreatic β-cells translocate to the nucleus under hyperglycemia, while the resultant nuclear Piezo1 is reversible by treatment in standard glucose concentration.
3. Piezo1 is involved in hypotonic swelling-induced depolarization in β-cells and mediates mechanical force-stimulated insulin secretion.
4. Piezo1 is important for glucose-stimulated Ca^{2+} signaling, membrane depolarization and GSIS.
5. Piezo1 controls large gene networks with, particularly Cocaine- and Amphetamine-Regulated Transcript (CART) and regulates hypotonic swelling-induced depolarization and insulin secretion.
6. β-cell specific *Piezo1* knockout male mice show an age-dependent effect on glucose utilization *in vivo* and GSIS *ex vivo*.

Paper III

Tcf7l2 controls expression of *Cacna2d1*/α2δ-1

TCF7L2 possesses the strongest genetic risk for T2D [194-196]. RNA-seq data indicates that one of the targeting genes by Tcf7l2 is the calcium channel subunit α2δ-1/*Cacna2d1* [14]. *Tcf7l2* silencing in INS-1 832/13 cells and rat islets results in a significant downregulation of the mRNA expression as well as the protein level of *Cacna2d1*/α2δ-1. The subunit α2δ-1 is primarily found in brain, heart and skeletal muscle [245] and here we confirm the presence of α2δ-1 in insulin-secreting cell lines and pancreatic islets. α2δ-1 displays a predominant expression in islets as compared to the other α2δ subtypes [246].

Silencing of *Cacna2d1* prevents trafficking of Cav1.2 to the plasma membrane and retains it in recycling endosomes

It has been well studied that α2δ-subunits regulate the trafficking of Cavα1-subunit to the plasma membrane (PM) in *Xenopus* oocytes and brain [140, 141, 247], but not yet in pancreatic cells. Pancreatic β-cells possess calcium channels including Cav1.2, Cav1.3, Cav2.1, Cav2.2, Cav2.3 and Cav3.1 [125]. Half of the whole Ca^{2+} currents are conducted by the L-type calcium channels (Cav1.2 and Cav1.3) [120].

Cav1.2 is considered the major subtype of L-type Ca^{2+}-channels in mouse β-cells [124]. Therefore, we checked the expression and localization of Cav1.2 after silencing of *Cacna2d1*. However, neither mRNA expression nor protein level of Cav1.2 is affected by the deficiency of *Cacna2d1*.

Since resident proteins are transported via sophisticatedly regulated pathways [248], we attempt to study the effect of α2δ-1 on Cav1.2 cellular distribution. The L-type Ca^{2+} channel α-subunit Cav1.2 is reported to be recycling between Rab11-recycling endosomes and PM [249, 250]. Therefore, we tested whether α2δ-1 influenced the trafficking of Cav1.2. As expected, the PM-located (Na^+/K^+-ATPase as the marker) Cav1.2 is significantly reduced and the amount in the recycling endosomes (Rab11 as the marker) is accordingly increased in *Cacna2d1*-silenced cells. Furthermore, overexpression of α2δ-1 induces the PM expression of Cav1.2. These findings indicate that α2δ-1 is responsible for the trafficking of Cav1.2 to the PM in β-cells. Regarding the regulation of Tcf7l2 on α2δ-1, the effect of Tcf7l2 on translocation of Cav1.2 was also tested, but the effects were inconsistent. Silencing of *Tcf7l2* fails to decrease the plasma membrane localization of Cav1.2. The indirect effect of Tcf7l2 on PM expression of Cav1.2 might be compensated by other target genes, the identity of which are uncertain at this point.

Silencing of *Cacna2d1* affects Ca^{2+} signaling and exocytosis

We then investigated whether changing the PM expression of Cav1.2 by interfering with α2δ-1 can affect Ca^{2+} signaling. Silencing of *Cacna2d1* leads to a significant drop of both depolarization-evoked and glucose-stimulated increases in $[Ca^{2+}]_i$ compared to control.

The drug gabapentin (GBP) is used clinically as an anti-epileptic treatment. The mechanism of action involves inhibition of α2δ subunits. As previously reported, long-term treatment (>24 h) with GBP reduces Ca^{2+} currents in dorsal root ganglion (DRG) neurons [251]. In the present study, pharmacological inhibition of α2δ subunits by GBP also results in markedly reduced K^+-stimulated $[Ca^{2+}]_i$ peaks in INS-1 832/13 cells. In line with this finding, both *Cacna2d1*-silencing or GBP treatment also decrease whole-cell Ca^{2+} currents in mouse islet β-cells. Clusters of Cav1.2 channels in COS1 cells can only be formed in the PM and manifest as functional voltage-gated channels when β-and α2δ-subunits are fully assembled [252]. Unlike the β-subunits, α2δ subunits affect both trafficking of Cav1.2 and Cav1.2-modulated Ca^{2+} currents [141]. Another intriguing observation suggests that Cav channel localization is important for the serious neurodegenerative disorder Huntington's disease. It has been suggested that Cav1.2 localization in the PM is regulated by the huntingtin-associated protein 1 (Hap1). The Hap1 protein is also present in β-cells and is essential for Ca^{2+} influx and insulin secretion [253]. Protein kinase C (PKC) also serves as a regulator for enhancing PM expression of Cav1.2

and by this mechanism facilitates macroscopic currents in a murine cardiomyocyte cell line (HL-1 cells) [254]. Hence, trafficking of Cav1.2 to PM is tightly controlled by various signalling pathways. Our data provide significant input in this respect in pancreatic β-cells.

Furthermore, our data corroborate previous reports to the effect that silencing of the diabetes gene *Tcf7l2* reduces $[Ca^{2+}]_i$ signalling evoked by either high K^+- or glucose. We conclude that Cav1.2 is modulated by α2δ-1 [141, 252] and α2δ-1 is only one of the multiple downstream targets of Tcf7l2, whereby the diabetes gene can regulate voltage-gated Ca^{2+} influx in β-cells.

We finally explored the effects of α2δ-1/Tcf7l2 on GSIS in which Ca^{2+} is the major triggering factor [39, 42]. As hypothesized, silencing of *Cacna2d1* significantly reduces GSIS and silencing of *Tcf7l2* even more so. However, double knockdown of both genes had the strongest effect on reducing GSIS. This might indicate that other target genes of Tcf7l2 (e.g. *ISL1*, *MAFA*, and *PDX1*) are involved [14].

Much to our surprise, treatment of GBP fails to influence insulin secretion. The exocytosis rate in INS-1 832/13 cells is unaffected by addition of GBP, which excludes the possibility of a confounding action by GBP directly stimulating the exocytotic machinery. Several cases about severe hypoglycemia from GBP-treated diabetic and non-diabetic patients have been reported, which is suggestive of an additional insulinotropic effect of GBP [255]. Nevertheless, at present, we have failed to identify a reasonable explanation. We assume that other GBP-related biological pathways interfere and compensate for the inhibition of insulin secretion exerted via affecting α2δ subunits.

Overexpression of α2δ-1 partially counteracts the effect of silencing *Tcf7l2* on Ca^{2+} signaling

To study whether the defect of Tcf7l2 could be rescued by α2δ-1, we overexpressed α2δ-1 in *Tcf7l2*-silenced cells. The reduced high glucose-stimulated $[Ca^{2+}]_i$ signalling by silencing *Tcf7l2* is significantly counteracted by overexpression of α2δ-1. This probably can be attributed to favouring a PM localization of Cav1.2. Therefore, increasing expression or activity of α2δ-1 is a means of counteracting the effects of dysfunctional *Tcf7l2* on Ca^{2+} signalling in the β-cell. Seemingly contradicting this, $[Ca^{2+}]_i$ is not further increased after overexpression of α2δ-1 in control cells. However, since the auxiliary subunits (α2δ, β and γ) are associated with the α1 subunits (Cav1 or Cav2) in a 1:1:1:1 ratio[127, 131], it is reasonable to assume that excess α2δ-1 has no positive effect on Cav channels assembly.

Overexpression of α2δ-1 stimulates both high K^+- and glucose-induced insulin secretion strongly, but it fails to improve the defective insulin secretion in *Tcf7l2*-silenced cells. How can this be explained? Deletion of Tcf7l2 has been reported to

result in a reduction of genes controlling exocytosis [14]. This we confirmed in our own setup, and qPCR analysis verified that silencing of *Tcf7l2* downregulates three SNARE complex-related genes Syt14, Stxbp1 and Vamp2. Likewise, silencing of *Cacna2d1* has similar effects on these genes except Vamp2. However, the downregulation of these genes by impaired *Tcf7l2* function can not be rescued by overexpressing α2δ-1. This explains why α2δ-1 fails to improve the impaired insulin secretion in *Tcf7l2*-silenced cells even though the Ca^{2+} signaling is normalized. Taken together, the complicated regulation of Tcf7l2 on the exocytotic machinery can be partially counteracted by overexpression of α2δ-1.

Highlights

1. Tcf7l2 controls mRNA and protein expression of the Cav channel auxiliary subunit *Cacna2d1*/α2δ-1.
2. The subunit α2δ-1 enhances the trafficking of Cav1.2 to PM, silencing of *Cacna2d1* results in Cav1.2 being retained in the recycling endosome.
3. Silencing of *Cacna2d1* reduces both high K^+- and glucose-induced Ca^{2+} signaling and impairs GSIS, whilst overexpression of α2δ-1 increases insulin secretion.
4. Overexpression of α2δ-1 improves the defective $[Ca^{2+}]_i$ in *Tcf7l2*-silenced cells but fails to affect the reduced gene expression of *Syt14*, *Stxbp1,* thereby it cannot fully reverse the defects caused by *Tcf7l2* silencing.

Paper IV

Expression of Cavγ4 is downregulated in islets in T2D

Cavα2δ, β, γ subunits are the three auxiliary components associated with the pore-forming Cavα1 subunits and among which, α2δ, β subunits are well studied to be pivotal for the trafficking of α1 subunits [140, 164, 256]. However, the role of γ subunits remains largely elusive in pancreatic β-cells. Among the eight isoforms, γ4 subunit was selected due to the significantly reduced expression in hyperglycemic human donors. Similar observations were made in type 2 diabetic rat/mouse models: Goto-kakizaki (GK) rats and *db/db* mice, but no alteration is observed in type 1 diabetic Akita mice. The expression of γ4 is also decreased by high glucose or palmitate treatment that mimics the diabetic condition. γ4 appears to be distributed in the cytosolic and membrane area in human islet β-cells. Since γ4 is physically

associated with Cav1.2 channels [129], it makes sense that γ4 distribution is consistent with the Cav1.2 expression pattern in pancreatic β-cells [249, 257].

Cavγ4 is required for GSIS and exocytosis

The altered expression of γ4 subunit in T2D human islets suggested us to further investigate its function in insulin release. Silencing of *CACNG4/Cacng4* in islets from human or rats result in clear suppression of GSIS. In line with this, depolarization-evoked increases in cell capacitance, reflecting β-cell exocytosis, are significantly decreased in *Cacng4*-silenced rat islet β-cells. Notably, both the 1st and 2nd phases of exocytosis (representing discharge of the readily releasable, and reserve pool of insulin granules, respectively) are dramatically reduced due to knockdown of *Cacng4*. Conversely, overexpression of γ4 improves the perturbed GSIS in diabetic human or GK rat islets and upregulates exocytosis in GK rat islet β-cells.

The importance of Ca^{2+} in GSIS has been repeatedly emphasized above, as well as by others [39, 42]. Therefore, we further explored whether γ4 is involved in β-cell Ca^{2+} homeostasis. Intracellular Ca^{2+} concentrations under either high K^+ or high glucose stimulation are remarkedly lowered in *Cacng4*-silenced INS-1 832/13 cells. By contrast, silencing of the gene family member *Cacng5* had no such effects. To exclude the possibility of contribution of Ca^{2+} from intracellular stores (e.g. ER, endosomes and lysosomes) [151, 258], voltage-gated Ca^{2+} currents were measured by patch clamp. As expected, silencing of *Cacng4* significantly reduced whole-cell Ca^{2+} currents in rat β-cells and INS-1 832/13 cells. In contrast, overexpression of γ4 enhanced voltage-gated Ca^{2+} influx in non-diabetic/T2D human and Wistar/GK rat β-cells. Taken together, these results demonstrate that γ4 affects intracellular Ca^{2+} concentrations by controlling influx from the extracellular space.

Cavγ4 controls L-type Ca^{2+} channel expression

To further study the specific pore-forming α1 subunits involved in the γ4-mediated Ca^{2+} influx, pharmacological inhibitors of Ca^{2+} channels were applied in Ca^{2+} currents measurements. Silencing of *Cacng4* fails to further reduce isradipine (L-type Ca^{2+} channel blocker)-inhibited Ca^{2+} currents but retains its suppressive effect on Ca^{2+} influx in the presence of a cocktail of non-L-type Ca^{2+} channel inhibitors. This strongly suggests the involvement of L-type Ca^{2+} channels. Abundant results from RNA-seq analyses, qPCR and immunoblotting reveal that γ4 positively regulates the expression of Cav1.2 and Cav1.3 (two of the main L-type Ca^{2+} channels in β-cells [120, 124]). Moreover, γ4 physically associates with Cav1.3 while we failed to achieve evidence for direct interaction between γ4 and Cav1.2.

However, such association between γ4 and Cav1.2 has been verified in HEK293 cells, in which Cav1.2 currents activation/inactivation are differentially modulated by γ4 in the presence/absence of α2δ-1 and β2 subunits [129]. Cav1.2 and Cav1.3 are firmly established as the main L-type channels regulating insulin secretion in rodent β-cells. Furthermore, their expression levels affect glycemic status in both human and mice [120, 126, 259]. Cavγ4 has no correlations with the other two L-type Ca^{2+} channels, Cav1.1 and Cav1.4, which is consistent with these two channels being important in skeletal muscle and retina, but not in pancreatic islet β-cells [116, 125].

Notably, silencing of *Cacng4* also downregulates α2δ-1 expression which modulates the trafficking of Cav1.2 to PM in β-cells [257], this provides an alternative means of α2δ-1 regulation other than Tcf7l2. Moreover, it points to γ4 being capable of altering PM expression of Cav1.2 which is critical for Cav1.2 Ca^{2+} currents [257, 260].

Taken together, all the evidence above suggests that γ4 manifests its role in Ca^{2+} influx and GSIS via the regulation of L-type Ca^{2+} channel expression (Cav1.2 and Cav1.3). The other auxiliary subunit α2δ-1 is shown to control Cav1.2 trafficking and to influence the β-cell stimulus-secretion coupling pathway in Paper III. These findings identify additional mechanisms for the auxiliary subunits and clarify how they affect β-cell function.

Cavγ4 is regulated by the transcription factor MafA

To fully understand the regulatory pathway involving γ4, the upstream regulator of γ4 was next explored. Microarray analysis of human islet transcription factors (TFs) provides a list of TFs that correlate with γ4. The β-cell maturation marker MafA [175-177] was confirmed by qPCR and immunoblotting as one of the positive controller candidates of γ4. MafA mediates the expression of γ4 by directly binding to its promoter and β-cell specific MafA ablation in mice ($MafA^{\Delta\beta cell}$) results in a strong reduction of γ4. Furthermore, overexpression of γ4 in $MafA^{\Delta\beta cell}$ islets improves Ca^{2+} influx and insulin exocytosis possibly via the rescued expression of Cav1.2 and Cav1.3. The L-type Ca^{2+} channel blocker (isradipine) and activator (Bay K) fail to exert their expected actions in modulating Ca^{2+} currents and $[Ca^{2+}]_i$ in β-cells from $MafA^{\Delta\beta cell}$ islets, which is in reminiscent of the findings in *Cacng4*-silenced cells. Therefore, we conclude that Cavγ4 is part of the MafA regulatory pathway and determines normal β-cell function.

β-cell viability remains unchanged in *Cacng4*-silenced cells compared to control cells, as evidenced by a series of apoptosis and proliferation assays (Cleaved Caspase-3 [261, 262] and P21 [263]). Interestingly, β-cell dedifferentiation is

induced after silencing of *Cacng4*, as indicated by the β-cell dedifferentiation marker Aldh1a3 (aldehyde dehydrogenase1A3) [264]). In accord with this, Aldh1a3 is suppressed by re-introducing γ4 in human islets. Several reports have shown that γ4 in brain is involved in neural differentiation [155, 265] and that Ca^{2+} signals control multiple developmental processes including differentiation [259, 266-268]. This clearly suggests that γ4 is required for maintaining the differentiation status of insulin-positive β-cells. Increasing evidence point to that it is dedifferentiation rather than β-cell death, causing β-cell failure in T2D [264, 269, 270]. Hence, restoring the differentiation of β-cells is a promising approach for treating T2D and γ4 may be the target in such therapy.

Highlights

1. Cavγ4 expression is downregulated in T2D patients and diabetic rodent models, as well as by *in vitro* glucotoxicity and lipotoxicity.
2. Cavγ4 regulates Ca^{2+} influx and GSIS via controlling L-type Ca^{2+} channels expression in both human and rodent islets.
3. Cavγ4 is directly controlled by β-cell transcription factor MafA.
4. Cavγ4 is a potential target for rescuing dedifferentiated β-cells in T2D.

Future perspectives

T2D is a complex disease resulting from the combined influence of genetic background, environmental impact and lifestyle. Understanding the linkage between the environmental input and gene regulatory pathways in insulin secretion from pancreatic β-cells is key for developing novel therapies to T2D. The *raison d'être* of the β-cell is the Ca^{2+}-dependent process of insulin exocytosis. This is according to the consensus view mediated by the closure of K_{ATP} channels and the activation of voltage-gated Ca^{2+} channels. This model has obvious shortcomings even though it has hypnotized the diabetes community for more than two decades. Studies in this thesis add novel data and knowledge to unravel the mechanism behind T2D development. Notably, the new findings of the involvement of mechanosensitive channel Piezo1 in regulating insulin release are revolutionary and will push on the old dogma. To completely understand the mechanosensing pathways, more detailed investigations are therefore required to pave ways for novel treatments of T2D.

Translocation of Piezo1 under different concentrations of glucose is of interest, but the function of the nuclear localization of Piezo1 in hyperglycemia requires further investigation. Importantly, it remains unclear whether Piezo1 distribution occurs before developing significant hyperglycemia or the other way around. The *db/db* mice show hyperinsulinemia at the age of 10 days and develop hyperglycemia at 8 weeks [271]. It is thus a good model to monitor Piezo1 distribution during the development of T2D. Knowing the cause-consequence link would clarify whether it is a good idea to prevent Piezo1 redistribution to the nucleus and thus rescue its normal function of mediating rhythmic action potential firing and insulin secretion.

Furthermore, we have provided initial clues to which regions of the Piezo1 protein are relevant for translocation, but a series of questions remain to be addressed: as one of the largest proteins, does Piezo1 need a chaperone for promoting translocation? Which is the domain harboring the translocation signal? Does it translocate by disintegrating into several parts or does the protein remain intact?

Piezo1 expression levels in neighboring glucagon-releasing α-cells are comparable to those in β-cells. However, α-cells show nuclear localization of Piezo1 when at rest under standard glucose concentration (10 mM), which is opposite to the situation in β-cells that reveal this pattern under conditions of intense stimulation. Does this indicate either an opposite function of Piezo1 in α-cells or suggest the

channel actively balances glucagon and insulin secretion? This prompts the importance to further investigate the multifaceted roles of Piezo1 in pancreatic cells other than β-cells.

The generation of the β-cell specific *Piezo1* knockout mouse model is a milestone for studying Piezo1 *in vivo*. In young *Piezo1* KO mice, glucose tolerance and insulin secretion are impaired. Surprisingly, these parameters are improved with increasing age in *Piezo1* KO mice. To better understand the age-dependent effect of missing Piezo1, single-cell RNA sequencing in islets from different ages of *Piezo1* KO mice would provide a broad knowledge of genes regulated by Piezo1.

Given the upregulatation of *Cartpt* after silencing of *Piezo1* in INS-1 832/13 cells, compensation of β-cell mass influencing factors might occur in *Piezo1* KO mice [242]. Therefore, examination of β-cell viability/proliferation and differentiation state would also be preferred in *Piezo1* KO mice. Cart (encoded by *Cartpt*) also facilitates the insulin exocytosis machinery [241], thus monitoring insulin secretion after regulating the expression of Cart (could also be other candidates as judged by the single-cell RNA-seq data) in β-cell *Piezo1*-deleted islets from different ages of mice is an alternative experiment to identify the functional factor behind the scenes.

We have demonstrated that β-cells are mechanosensitive and Piezo1 exerts a key role in sensing glucose-induced swelling to modulate β-cell function. This provides revolutionary new insights into how the β-cell works and how novel therapies against T2D could be developed. Recently, magnetic forces applied to nanoparticle-labeled Piezo1 has been revealed to efficiently facilitate Piezo1 activation *in vitro* [272]. We have previously used a dynamic magnetic field generator to control the movement of superparamagnetic nanoparticles (SPIONs) and this innovative approach reversibly evokes Ca^{2+} responses by mechanic stimuli in the insulin-secreting cell line [273]. Using external magnetic fields to control Piezo1 activity, and consequently insulin secretion, is a feasible and interesting approach. Ultrasound application has been confirmed to activate heterologous and endogenous Piezo1 to initiate Ca^{2+} influx in primary neurons [274]. These reports indicate novel ways to apply controlled mechanical force stimulation targeting Piezo1 in β-cells, and is a promising modality for restoring β-cell function and treating T2D. However, to make it possible, we need to solve a series of problems, for instance, identifying the electrical gating domain and the mechanosensing region of Piezo1 in β-cells, to optimize control of mechanical force, etc.

Finally, in this thesis, we identified that MafA regulates the auxiliary subunit γ4 and that the subunit is involved in controlling β-cell differentiation. β-cell failure caused by de-differentiation plays a vital role in the development of T2D [264, 269, 270]. Our data show that the expression of γ4 in islets from donors with T2D is significantly reduced. Therefore, increasing γ4 levels to restore the differentiation of β-cells is a promising approach for treating T2D. Nevertheless, we need to further

understand the function of γ4 in this respect, Cavγ4 knockout mice [275] is a suitable model to investigate β-cell differentiation state and related β-cell function.

The findings in this thesis convincingly challenge the consensus model for glucose-stimulated insulin secretion and provide unequivocal evidence supporting a close to revolutionary view on the β-cell stimulus-secretion coupling involving the mechano-sensor Piezo1. The new concept of β-cell dedifferentiation in β-cell dysfunction is confirmed, and, surprisingly, the Cav channel subunit γ4 partakes in this reaction. Collectively, these findings open the door to several innovative approaches to finding new therapeutic strategies to combat T2D.

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Yingying Ye is a biomedical graduate from Lund University. Her main research interest is to investigate islet pathophysiology in type 2 diabetes. The focus on her thesis work was to explore the novel insulin secretion pathway involving mechanosensitive channel Piezo1 in pancreatic β-cells and the roles of Ca^{2+} channel subunits in regulating β-cell function.

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