



THE SUCCINATE-SUCNR1 AXIS AND ITS IMPACT ON BETA-CELL FUNCTION: AN INTEGRATIVE STUDY UNCOVERING NOVEL INSIGHTS INTO INSULIN SECRETION REGULATION AND DIABETES PATHOGENESIS

Joan-Martí Sabadell i Basallote

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The Succinate-SUCNR1 Axis and Its Impact on Beta-Cell Function

An Integrative Study Uncovering Novel
Insights into **Insulin Secretion**
Regulation and **Diabetes**
Pathogenesis

Joan Sabadell-Basallote

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DOCTORAL THESIS

Supervised by:

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CERTIFY THAT

The present doctoral thesis, entitled **“The Succinate-SUCNR1 Axis and Its Impact on Beta-Cell Function: An Integrative Study Unveiling Novel Insights into Insulin Secretion Regulation and Diabetes Pathogenesis”**, presented by **Joan-Martí Sabadell i Basallote** has been conducted under our direct supervision at the Pere Virgili Health Research Institute and the Department of Medicine and Surgery of this university and fulfills the requirements for the award of the Doctorate degree.

This certification is signed in Tarragona on June 15, 2023, for official record and initiation of the necessary proceedings.

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Before delving into this thesis, I would like to take a moment to express this truth: I did not walk this path alone. Every page, each eureka moment, and all hurdles overcome represent the collective efforts of a people constellation whose immeasurable influence is etched in every word.

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Abbreviations

A

α-KG α-ketoglutarate

AC Adenylyl cyclase

AcAc Acetoacetyl

ADA American Diabetes Association

ADP Adenosine diphosphate

AGE Advanced glycation end product

AMPK Adenosine mono-phosphate-activated protein kinase

ANOVA Analysis of variance

AT Adipose tissue

ATAC-seq Assay for transposase-accessible chromatin using sequencing

ATM Adipose tissue macrophage

ATP Adenosine triphosphate

AUC Area under the curve

B

BAT Brown adipose tissue

BMI Body mass index

BSA Bovine serum albumin

C

C-peptide Connecting peptide

[Ca²⁺]_i Intracellular Ca²⁺ concentration

cAMP Cyclic adenosine monophosphate

cDNA Complementary DNA

cESA *Cis*-epoxysuccinic acid

ChIP-seq Chromatin immunoprecipitation and sequencing

CM Conditioned media

CNS Central nervous system

CoA Coenzyme A

CREB cAMP response element-binding protein

Ct Cycle threshold

D

DAG Diacylglycerol

DIC Dicarboxylate carrier

DIO Diet-induced obesity

dNTP Deoxyribonucleotide triphosphate

DPBS Dulbecco's phosphate-buffered saline

DPP-4 Dipeptidyl peptidase 4

E

EASD European Association for the Study of Diabetes

EC₅₀ Half maximal effective concentration

ECM Extracellular matrix

ELISA Enzyme-linked immunosorbent assay

ER Endoplasmic reticulum

ERK1/2 Extracellular signal-regulated kinase 1/2

ETC Electron transport chain

F

FACS Fluorescence-activated cell sorting

FAD Flavin adenine dinucleotide

FBS Fetal bovine serum

FFAR1 Free-fatty acid receptor 1

fl Floxed

FOXA2 Forkhead box protein A2

FOXO1 Forkhead box protein O1

Fura-2 AM Fura-2 acetoxymethyl ester

G

G6P Glucose-6-phosphate

GABA γ-aminobutyric acid

GCGR Glucagon receptor

GDM Gestational diabetes mellitus

GDP Guanosine diphosphate

GIP Glucose-dependent insulinotropic polypeptide

GIPR GIP receptor

GK Glucokinase

GLP-1 Glucagon-like peptide 1

GLP-1R GLP-1 receptor

GLUT Glucose transporter

GPCR G-protein-coupled receptor

GSIS Glucose-stimulated insulin secretion

GSK-3 Glycogen synthase kinase-3

GTP Guanosine triphosphate

GTT Glucose tolerance test

H

H&E Hematoxylin and eosin staining

HbA1c Glycated hemoglobin

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFD High fat diet

HIF-1α Hypoxia-inducible factor-1α

HMG Hydroxy-3 methylglutaryl

HOMA-B Homeostasis model assessment of β-cell function

HOMA-IR Homeostasis model assessment of insulin resistance

hTERT Human telomerase reverse transcriptase

I

i.p. Intraperitoneal

IDF International Diabetes Federation

IEQ Islet equivalent

IFG Impaired fasting glucose

IGT Impaired glucose tolerance

IHC Immunohistochemistry

IIGI Isoglycemic intravenous glucose infusion

IL-1 β Interleukin 1 beta

Ins1 Mouse insulin I gene

IP₃ Inositol trisphosphate

IR Insulin receptor

IRB Islet Regulome Browser

IRS Insulin receptor substrate

ISR Insulin secretion rate

ITT Insulin tolerance test

J

JNK c-Jun N-terminal kinase

K

K_{ATP} ATP-sensitive potassium

K_m Michaelis constant

KO Knockout

KRPB Krebs-Ringer-phosphate-HEPES buffer

L

LH Locke-HEPES buffer

M

M-PER Mammalian protein extraction reagent

MAFLD Metabolic-associated fatty liver disease

MAPK Mitogen-activated protein kinase

MCT Monocarboxylate transporter

MTT Meal tolerance test

N

NaCT Na⁺/citrate cotransporter

NAD Nicotinamide adenine dinucleotide

NaDC Sodium-dependent dicarboxylate transporters

NADP Nicotinamide adenine dinucleotide phosphate

NCD Normal chow diet

NF- κ B Nuclear factor κ -light-chain-enhancer of activated B cells

NGT Normal glucose tolerant

NKX2.2 NK2 homeobox 2

NKX6.1 NK6 homeobox 1

NLRP3 NACHT, LRR, and PYD domains-containing protein 3

O

OAT Organic anion/dicarboxylate transporter

OGIS OGTT-derived insulin sensitivity index

OGTT Oral glucose tolerance test

P

PBS Phosphate-buffered saline

PC Pyruvate carboxylase

PCR Polymerase chain reaction

PDH Pyruvate dehydrogenase

PHD Prolyl hydroxylase domain

PI3K Phosphatidylinositol 3-kinase

PIP₂ Phosphatidylinositol 4,5-diphosphate

PKA Protein kinase A

PKC Protein kinase C

PLC Phospholipase C

PP Pancreatic polypeptide

PTM Post-translational modification

Q

qPCR Quantitative real-time PCR

R

RCF Relative centrifugal force

RER Rough endoplasmic reticulum

RNA-seq RNA sequencing

ROS Reactive oxygen species

RT Room temperature

S

SD Standard deviation

SDH Succinate dehydrogenase

SEM Standard error mean

SGLT2 Sodium-glucose cotransporter 2

SIRT5 Sirtuin 5

SLC Solute carrier

SNP Single nucleotide polymorphism

SUCNR1 Succinate receptor 1

***Sucnr1*-βKO** β-cell-specific *Sucnr1* knockout

SUR Sulfonylurea receptor

SV40LT SV40 large T antigen

T

T1D Type 1 diabetes

T2D Type 2 diabetes

TAE Tris-acetate-EDTA

TBS-T Tris-buffered saline with Tween 20

TCA Tricarboxylic acid

TNF-α Tumor necrosis factor-α

U

UCP1 Uncoupling protein 1

UPR Unfolded protein response

V

VDAC Voltage-dependent anion channel

VGCC Voltage-gated Ca²⁺ channel

VLDL Very low-density lipoprotein

vs. Versus

W

WAT White adipose tissue

WHO World Health Organization

WT Wild-type

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Abstract

Pancreatic β -cell dysfunction constitutes a fundamental characteristic of type 2 diabetes (T2D), and novel regulators of insulin secretion are desirable. This doctoral thesis presents an integrative and translational study of the succinate-succinate receptor 1 (SUCNR1) axis in β -cells. Succinate, a tricarboxylic acid cycle substrate, has emerged as a metabolite with broader functions beyond energy metabolism. While traditionally associated with hypoxia, tissue damage, and metabolic disorders, current research has revealed a connection between succinate response and SUCNR1 activation and physiological processes, including exercise and the fasted-to-fed transition. This reshapes the paradigm from considering SUCNR1 solely as a sensor for tissue damage to recognizing it as a versatile systemic metabolic signal modulator. Within β -cells, intracellular succinate has been associated with insulin biosynthesis and release. However, its extramitochondrial and extracytosolic roles in β -cells are still unexplored. Furthermore, SUCNR1 expression has been observed in the pancreas and human islets, indicating a potential instructive role for the succinate-SUCNR1 axis in islet physiology.

Our study results provide evidence that SUCNR1 is prominently expressed in β -cells and exhibits up-regulation in hyperglycemic states, including elevated SUCNR1 levels in human islets during obesity and T2D. Succinate, acting as a hormone-like metabolite associated with food intake, significantly influences insulin secretion through SUCNR1 engagement. β -cells effectively sense extracellular succinate under high glucose, thereby enhancing insulin release through a SUCNR1-Gq-PKC-dependent mechanism.

To further elucidate the significance of SUCNR1, we conducted a comprehensive examination of β -cell-specific *Sucnr1* knockout mice and observed glucose tolerance impairment and insulin secretion deficiency in the context of a high-fat diet. These alterations occurred independently of any changes in β -cell mass or insulin sensitivity, highlighting the essential nature of SUCNR1 in preserving insulin secretion in the face of diet-induced insulin resistance.

Moreover, our investigation extended to subjects with prediabetes who exhibited concurrent hyperinsulinemia. These individuals demonstrated an enhanced nutritional-related succinate response, assessed through oral glucose challenges and isoglycemic intravenous glucose infusions. Importantly, this augmented succinate

response strongly correlated with the potentiation of insulin secretion during intravenous glucose administration.

Collectively, these data demonstrate that the succinate-SUCNR1 axis is required for preserving insulin secretion in hyperglycemia and is partly regulated by β -cells in an autocrine fashion. These data support a novel GPCR-mediated amplifying pathway for insulin secretion that is particularly relevant to the hyperinsulinemia of people with prediabetes.

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UNCOVERING NOVEL INSIGHTS INTO INSULIN SECRETION REGULATION AND DIABETES PATHOGENESIS

Joan-Martí Sabadell i Basallote

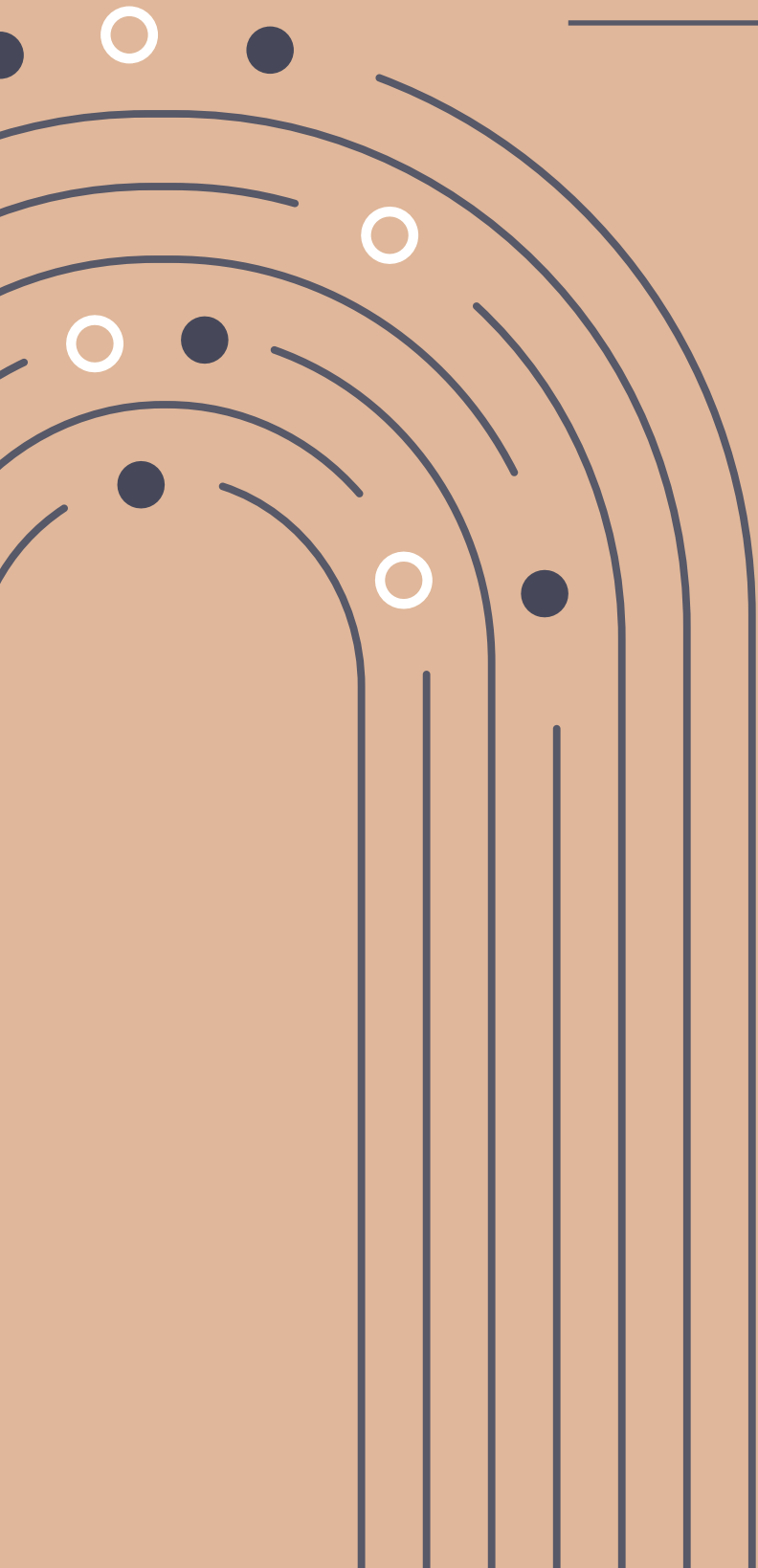
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Introduction

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1.1. Regulation of Glucose Homeostasis

Glucose homeostasis, the delicate balancing act that ensures optimal blood glucose levels, is a fundamental physiological process integral to the survival and well-being of organisms. The ability to maintain appropriate glucose concentrations in the bloodstream has been pivotal to the evolution and success of many species, particularly during times of food scarcity or prolonged fasting¹.

Glucose, a versatile and efficient energy source, fuels cellular activities by being readily converted into adenosine triphosphate (ATP), the primary energy currency of organisms, through either cellular respiration or fermentation^{2,3}. In mammals, specific organs, such as the brain, heavily rely on glucose-derived ATP to carry out their vital functions^{4,5}. In order to provide organs with glucose, intricate mechanisms have evolved to regulate blood glucose levels in response to fluctuations caused by food intake and energy consumption^{5,6}.

At the core of glucose homeostasis lies a sophisticated interplay between pancreatic hormones, primarily insulin and glucagon, which act in concert to maintain equilibrium. Insulin lowers blood glucose levels by facilitating glucose uptake and utilization in tissues like the liver and muscle, while glucagon serves as a counter-regulatory hormone^{5,7}. This delicate balance is influenced by a myriad of factors, including diet, exercise, and multiple physiological signals⁸⁻¹¹.

Insulin's pivotal role in glucose homeostasis is further highlighted by the severe consequences that arise from disruptions in its production or secretion, leading to uncontrolled blood glucose levels, which occur in diabetes^{7,12}. Recognized as a global epidemic, diabetes is among the most pervasive diseases, with its incidence escalating relentlessly across the globe¹³⁻¹⁵. Therefore, deepening our comprehension of the processes underlying insulin secretion presents a critical step toward the development of innovative treatments, potentially improving the lives of millions afflicted with diabetes and related metabolic disorders around the world.

In recent years, breakthroughs in unraveling the complex interplay between various hormones, cellular signaling pathways, and metabolic processes have shed light on the regulation of glucose homeostasis in physiology. In this section, the intricacies of glucose regulation are delved into, and both well-established knowledge and the latest discoveries are woven together to offer a comprehensive understanding of this vital physiological process.

1.1.1. Anatomy of the Pancreas

Originating from ventral and dorsal buds in the distal foregut, the pancreas is a unique glandular organ that is instrumental in regulating glucose homeostasis by hormone secretion, as well as managing digestion through its dual endocrine and exocrine roles, respectively. At the macroscopic level, the pancreas exhibits a unique anatomical structure comprising the head, body, and tail, with a lobular arrangement in its parenchyma¹⁶. The organ also incorporates a series of discharge ducts, essential for its digestive function. Acini, small sac-like structures producing and storing digestive enzymes, are interconnected by ducts that enable the drainage of their contents into a central tube (i.e., the pancreatic duct). This duct extends from the pancreas' tail and traverses the entire length of the organ before eventually converging with the common bile duct and entering the duodenum through the ampulla of Vater^{17,18}. In addition, many individuals possess an accessory pancreatic duct that empties into the duodenum *via* the minor duodenal papilla^{17,18} (**Figure 1**).

At the microscopic level, the pancreas comprises two fundamental elements: the exocrine and the endocrine compartments¹⁸. On the one hand, approximately 98% of the pancreas mass consists of the exocrine cells, which its function involves the production of digestive enzymes for proteins, fats, carbohydrates, and nucleic ac-

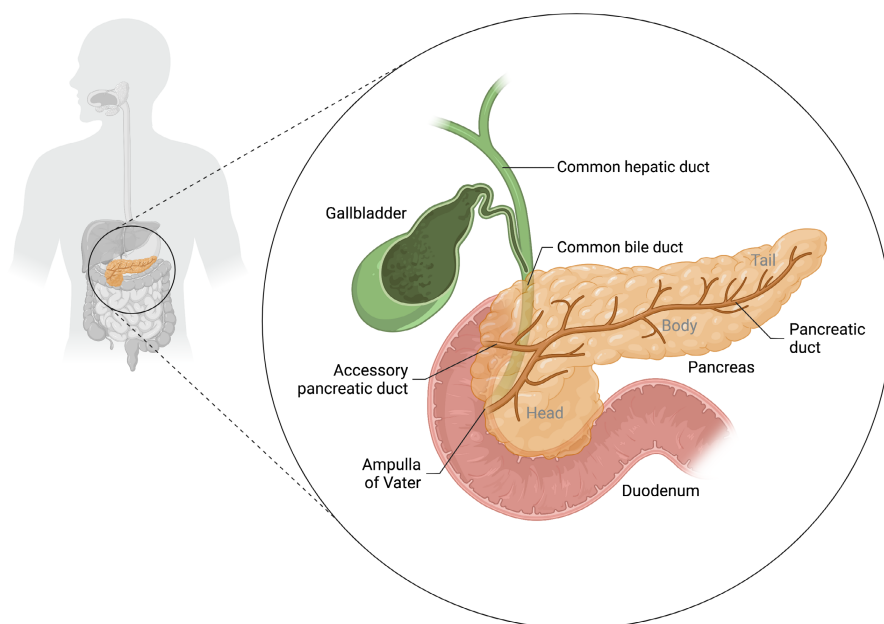


Figure 1. Overview of the Human Pancreas Anatomy. Comprehensive view of the human pancreas, detailing its anatomical features and location in relation to neighboring organs.

ids^{18,19}. These enzymes are synthesized by acinar cells and then exported by exocytosis into the ducts, composed of ductal cells and, to a lesser extent, goblet cells^{20,21}. Additionally, the ductal and goblet cells secrete bicarbonate and mucin, respectively^{22,23}. Together, these enzymes and substances make up the iso-osmotic and alkaline pancreatic juice, which enables the breakdown of food and the subsequent absorption of nutrients into the body and, therefore, is critical for ensuring the seamless operation of the digestive system^{22,24}. On the other hand, the endocrine component accounts for approximately 1-2% of the entire organ and is organized into **pancreatic islets** (also referred to as islets of Langerhans), which are clusters of various cell types that secrete a variety of important hormones into the bloodstream^{7,25}. These islets are scattered throughout the pancreas parenchyma, allowing for close anatomical and functional connections with exocrine cells²⁵.

1.1.2. Structure and Function of Pancreatic Islets

The discovery of pancreatic islets was made in 1869 by a 21-year-old medical student named Paul Langerhans^{26,27}. However, their function remained unknown until the early 20th century, when several researchers studied the pancreas to identify its role in glucose homeostasis. Furthermore, other researchers reported changes in the islet's structure during this time, all before insulin's discovery in 1921²⁷. Nowadays, it is known that each islet in the pancreas, which can number anywhere from 1 to 15 million in humans, has its own blood supply, presents autonomic innervation, and contains thousands of highly-specialized cells that produce hormones^{28,29}. These hormone-producing cells are categorized into five different types based on the primary hormone they release: α -cells (which secrete glucagon), β -cells (insulin and amylin/islet amyloid polypeptide), δ -cells (somatostatin), γ -cells or pancreatic polypeptide (PP) cells, and the rare ϵ -cells (ghrelin)²⁵ (**Figure 2**).

The hormones produced by the islet cells play a crucial role in preserving the balance of energy substrates and maintaining metabolic homeostasis. Insulin, a vital anabolic hormone, stimulates the storage of metabolic substrates, while glucagon, with its catabolic properties, encourages the mobilization and oxidation of energy reserves. The release of these hormones is a tightly regulated and complex process that depends on the nutritional state, various physiological factors, and the crosstalk between the different cell types that compose islets and several organs in the body²⁵. For instance, insulin secretion is mainly activated by a postprandial increase in glucose concentrations, further enhanced by specific amino acids, fatty acids, and hormones produced in α -cells and the gastrointestinal tract. In contrast,

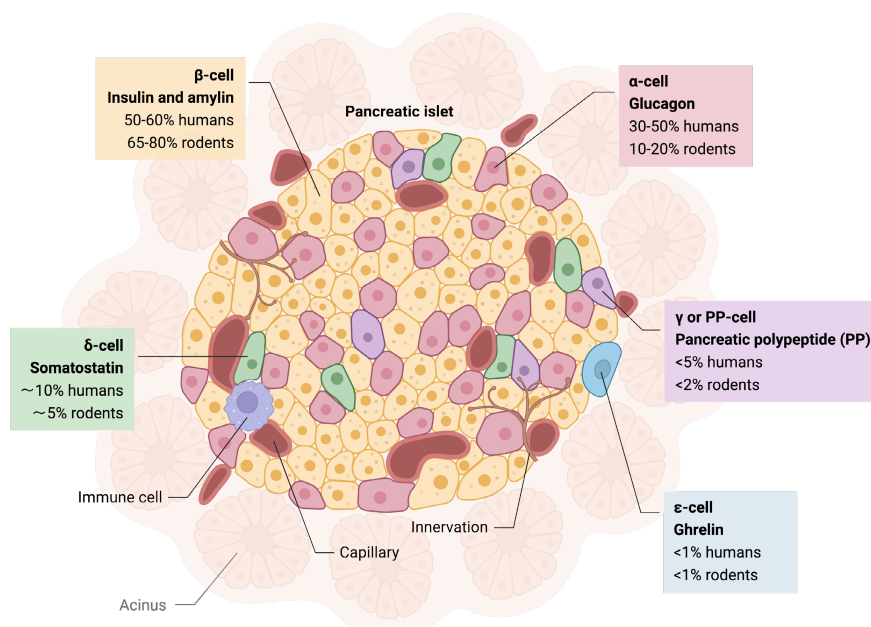


Figure 2. Cross-Sectional Visualization of a Pancreatic Islet. Each cell type within the pancreatic islet, the corresponding cellular proportions in humans and rodents, and the distinctive hormones they synthesize are detailed.

glucagon secretion is inhibited by elevated glucose and insulin levels but stimulated by fasting-induced rises in amino acids and fatty acids^{11,25}. As a potent paracrine inhibitor, somatostatin suppresses the secretion of both glucagon and insulin^{11,30}. Amylin inhibits glucagon secretion, delays gastric emptying, and enhances satiety¹¹. Ghrelin stimulates appetite, though it is mainly produced by enteroendocrine cells in the stomach^{11,25}, while PP acts as an appetite suppressant and promotes pancreatic secretion of digestive enzymes¹¹.

Research involving laboratory rodents, such as mice (*Mus musculus*) and rats (*Rattus norvegicus*), has significantly contributed to the study of islet biology. Islets in these animals exhibit a well-defined structure in histological sections and a heterogeneous proportion of endocrine cells, with β -cells comprising 65-80% of the islet's cells, surrounded by a layer of other endocrine cells, including α -cells (10-20% of the cells), δ -cells (approximately 5% of islet cells), γ -cells (approximately 2% of cells), and ϵ -cells (less than 1% of cells)³¹⁻³⁵. However, a three-dimensional analysis of islet distribution showed that the α -cell mantle around the β -cell core is non-continuous, despite appearing regular in many tissue sections³⁶. Islet size can vary significantly in rodents, ranging from small islets containing ten or fewer cells to larger islets composed of thousands of cells³¹.

Variations in the distribution of islets in the pancreas of rodents have also been investigated, with a report indicating that more islets are present in the tail compared to the head³⁷. However, other studies showed that the size and distribution of islets are comparable in different regions of the adult mouse pancreas³¹. While γ -cells are more frequently found in the head, the tail region of the pancreas has a higher concentration of α -cells^{38,39}.

Compared to rodent islets, human islets exhibit a relatively higher percentage of α -cells and fewer β -cells and do not have a well-defined distribution of endocrine cells^{34,40}. The cellular composition of human islets is highly heterogenous, comprising approximately 50-60% β -cells, 30-50% α -cells, nearly 10% δ -cells, less than 5% γ -cells, and the rare ϵ -cells (less than 1%)^{35,41,42}. The size of human islets varies greatly, with an approximate diameter of 50-500 μm and an average of 1500 cells per islet. Small islets are mainly composed of β -cells, while large islets have intertwined α - and β -cells³¹. The differences in endocrine cell ratios among individuals are accompanied by heterogeneity in β -cell mass^{29,43}, whereas differences in endocrine cell distribution across pancreatic regions are relatively insignificant⁴⁴. Notwithstanding, there is an anomaly in the posterior lobe of the pancreatic head area where γ -cells are notably abundant^{45,46}.

Interestingly, research on the structure of islets during human development indicates that the arrangement of endocrine cells in human embryos is similar to that of adult mice⁴⁷. Accordingly, at 12-14 weeks of gestation, human endocrine cells are found in clusters, with β -cells forming a core and α and δ -cells surrounding them. However, by the 18th week of gestation, the peripheral α and δ -cells separate from the β -cell core and form adjacent homogenous islets^{47,48}. These homogenous islets are presumed to re-integrate after the 22nd week, forming the configuration observed in adult islets⁴⁸. This demonstrates the dynamic nature of islet development during fetal growth and highlights the differences in hormonal expression between human fetal and adult islets.

In addition to endocrine cells, islets are composed of other entities and cells that support their function, which have essential yet not fully understood roles in islet homeostasis. These components serve as a scaffold for islets and provide nutrients, soluble factors, and signals that influence islet cell health and function⁴⁹. Vascular cells are present in the endocrine parenchyma, forming capillary tubes that link the islets to the systemic circulation^{50,51}. Unlike rodent islets that have highly vascularized capillaries, human islets have much lower vascular density^{52,53}. Although the

understanding of *in vivo* human islet vascularity and blood flow remains limited due to noticeable experimental limitations, a recent study suggested that islet microcirculation may not be unidirectional⁵².

Moreover, neuronal processes and immune cells are also found within human islets and provide signals influencing islet hormone secretion^{54,55}. For instance, neural projections extend into the islets from nerves outside the pancreas and may facilitate their regulation by the central nervous system (CNS)⁵⁴. In addition, the parenchymal and vascular components are surrounded by a network of macromolecules composing the extracellular matrix (ECM), where fibroblasts are the principal cells producing the ECM elements⁴⁹. The islet ECM composition is unique, made of collagens, heparan sulfate proteoglycans, laminin, elastin, fibronectin, and polysaccharides, among others^{49,56}, and has been proposed to provide a polarized microdomain to promote endocrine granule fusion. The ECM also influences islet cells' phenotype and function, signaling primarily through integrins^{56,57}. However, more research on human islets is required to understand the potential functions of the islet ECM.

An overview of a pancreatic islet in a relatively steady physiology state has been discussed. However, it is essential to recognize that the islet is a dynamic microscopic organ that undergoes adaptations in terms of its structure, cell composition, and function, which are influenced by various physiological (e.g., metabolic changes during pregnancy and aging) and pathophysiological factors (e.g., alterations that occur during obesity, insulin resistance, and diabetes)^{11,25}.

It is worth noting that investigating human islets or the whole pancreas for scientific purposes has multiple inherent limitations due to various factors, such as age or genetic disparities between donors, the acquisition or processing of the specimens, and the rapid changes after death. Additionally, the inability to control donors' physiology or pathophysiology before pancreas procurement can make it challenging to draw definitive mechanistic explanations. It is also unsafe to biopsy the pancreas in living individuals, except during particular surgical procedures. However, despite the abovementioned caveats, studying human islets is essential for a comprehensive understanding of the role of islets in various forms of diabetes. Moreover, it is important to remember that there are significant differences between human and nonhuman pancreatic islets, such as cell composition, arrangement, proliferation rates, and regulation and expression of crucial genes^{25,33}. Furthermore, despite significant advances in clinical imaging modalities and attempts to target human β -cells *in vivo*⁵⁸, there is still no reliable and noninvasive imaging of

functional β -cell mass. As such, a combination of biomarkers and postmortem tissue analyses remains the primary means for understanding how the islet changes over the human lifespan and with disease²⁵.

Overall, the proper function of the pancreatic islets is crucial for maintaining metabolic homeostasis and preventing the onset of diseases, primarily diabetes^{11,25}.

1.1.3. Insulin: Discovery, Structure, and Biosynthesis

The discovery of insulin in 1921 was a momentous landmark in the history of physiology and diabetes research, attributed to the consistent efforts and commitment of physicians Banting, Best, and Macleod. Using intravenous administration of pancreatic saline extracts on diabetic dogs, they identified these extracts' role in reducing blood glucose levels. One year later, the first human trials began, with a 14-year-old boy with diabetes showing extraordinary improvement after receiving a pancreatic beef isolate. The active component from these extracts was coined insulin, and production of porcine insulin began shortly thereafter for therapeutical purposes. Insulin's discovery not only revolutionized the treatment of diabetes but also opened up new avenues for research and understanding of metabolic disorders. As a recognition for their contribution, the Nobel Prize was awarded to Banting and Macleod in 1923^{59,60}.

During the 20th century, seminal studies provided insights into the nature and structure of insulin⁵⁹. Notably, insulin holds a special place in the history of molecular biology, as it was the first protein ever to be sequenced by Sanger in the 1950s⁶¹. This hormone is encoded in the short arm of chromosome 11 in humans and translated into a peptidic precursor (i.e., **preproinsulin**) in the rough endoplasmic reticulum (RER). Preproinsulin comprises 100 amino acids and contains a signal peptide, the B chain, the connecting (C) peptide, and the A chain⁶². The signal peptide's elimination by the signal peptidase in the RER⁶³ produces **proinsulin**, which takes on its unique three-dimensional configuration forming disulfide bonds by oxidation⁶². Through vesicular transportation, proinsulin is transferred to the Golgi complex, where its aqueous environment enriched with zinc and calcium encourages the formation of soluble zinc-containing proinsulin hexamers⁶⁴. Outside the Golgi complex, in immature secretory vesicles, prohormone convertases and carboxypeptidase E process proinsulin to yield insulin and C-peptide⁶². Mature insulin consists of A and B chains with 21 and 30 amino acids, respectively, linked by disulfide bridges, and it has a molecular weight of 5802 Da, with an isoelectric point of pH 5.5⁶⁵. The

A chain comprises an N-terminal helix and an anti-parallel C-terminal helix, while the B chain has a central helical segment⁶⁴. Eventually, when mature granules fuse with the plasma membrane, **insulin**, and **C-peptide** are released via **exocytosis** in an equimolar proportion into circulation⁶⁵ (**Figure 3**).

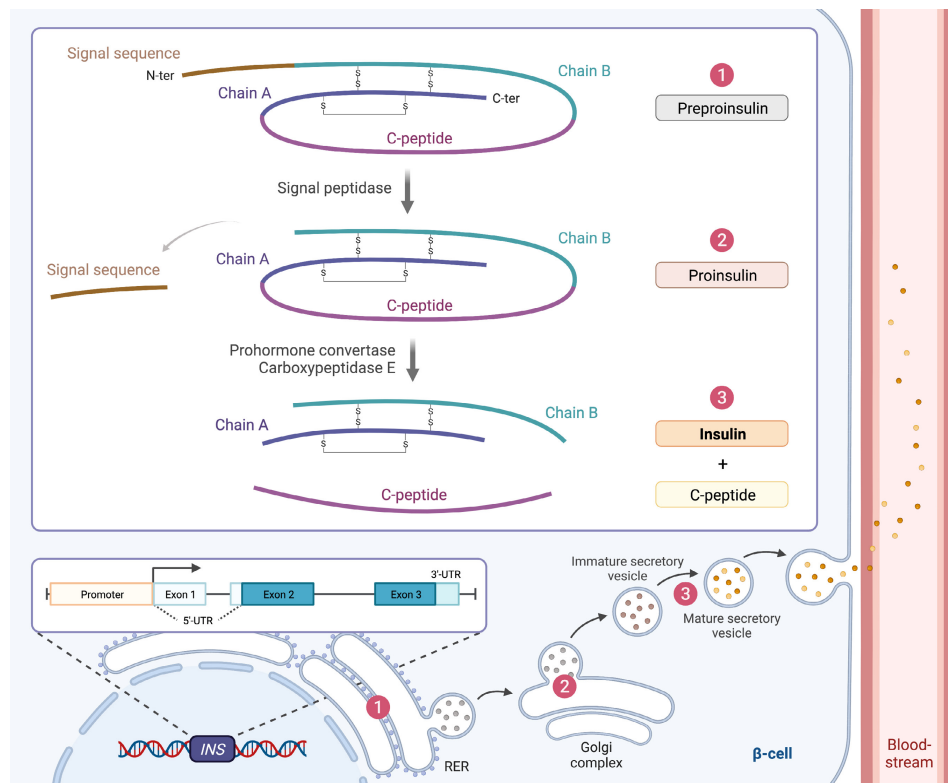


Figure 3. Schematic Illustration of Insulin Synthesis within the β-Cell. The depicted process details a three-step transformation: from preproinsulin to proinsulin and finally into mature insulin. The integral cellular compartments involved in this metabolic pathway are also outlined. First, the rough endoplasmic reticulum (RER) is the site for initial protein synthesis. Then, proinsulin is transported to the Golgi complex, where it undergoes further post-translational modifications. Post-Golgi, proinsulin is packed into immature secretory granules. As these granules mature, proinsulin is cleaved to form insulin and C-peptide, which are then stored within the mature secretory granules, poised for regulated release in response to stimuli. C-peptide: connecting peptide; *INS*: human insulin gene; UTR: untranslated region.

1.1.4. The Regulation of Insulin Secretion by the β-Cell

Following a meal, the rise in circulating glucose levels initiates insulin secretion from β-cells, a process that unfolds in two distinct phases^{11,25,66}.

The **first phase** involves the **triggering pathway**, set in motion by glucose catabolism. This phase results in rapid insulin secretion, demonstrated by a sharp peak

in insulin levels followed by a swift decline within 10-20 minutes. Notably, around 1% of readily releasable insulin granules are mobilized within this phase, signifying the triggering pathway's pivotal role in a prompt, albeit transient, insulin response. Conversely, the **second phase** is defined by **amplifying pathways**, which ensure prolonged and sustained insulin secretion. This phase spans a few hours and is characterized by a steady mobilization of insulin-containing granules from the β -cells' internal reserves to the cell surface, leading to continued insulin release^{11,25,66}. Therefore, while the triggering pathway underpins the immediate insulin response, the amplifying pathways, operational during the second phase, are essential in maintaining an extended insulin secretion, contributing to stable post-prandial blood glucose levels^{11,25,66}.

To gain a complete perspective on insulin secretion, it is essential to differentiate between the mechanisms that elicit the amplifying response. On the one hand, the **metabolic-amplifying pathway** maintains insulin secretion through intracellular metabolic reactions that are not associated with increasing intracellular Ca^{2+} levels. On the other hand, the **neurohormonal-amplifying pathway**, which encompasses the interplay of hormones, neurotransmitters, and energy substrates with cell receptors, further fine-tunes the insulin secretion process^{11,67,68} (**Figure 4**).

1.1.4.1. Glucose and Nutrient Metabolism Control on Insulin Secretion

Glucose-stimulated insulin secretion (GSIS) involves synchronizing various signals, including metabolic, electrical, and chemical cues within the β -cell^{35,69}. A distinguishing feature between human and rodent β -cells in GSIS is the predominant employment of glucose transporter (GLUT) 1 for facilitated glucose diffusion, as opposed to the GLUT2 transporter in rodents⁷⁰⁻⁷². However, the subsequent stages in the glucose-triggering pathway are believed to be similar in nonhuman and human β -cells²⁵. This pathway is activated by glucose metabolism, leading to **ATP production** and the closure of ATP-sensitive potassium (K_{ATP}) channels on the plasma membrane. The resulting **depolarization** of the cell membrane activates voltage-gated Ca^{2+} channels (VGCCs), increasing intracellular Ca^{2+} levels and prompting insulin secretion¹¹.

The rate of glucose metabolism holds a key position in the insulin secretion response, and it is governed by different coupling factors linked to glucose transport into the β -cell and glucose catabolism. **Glucokinase (GK)** or hexokinase IV plays a

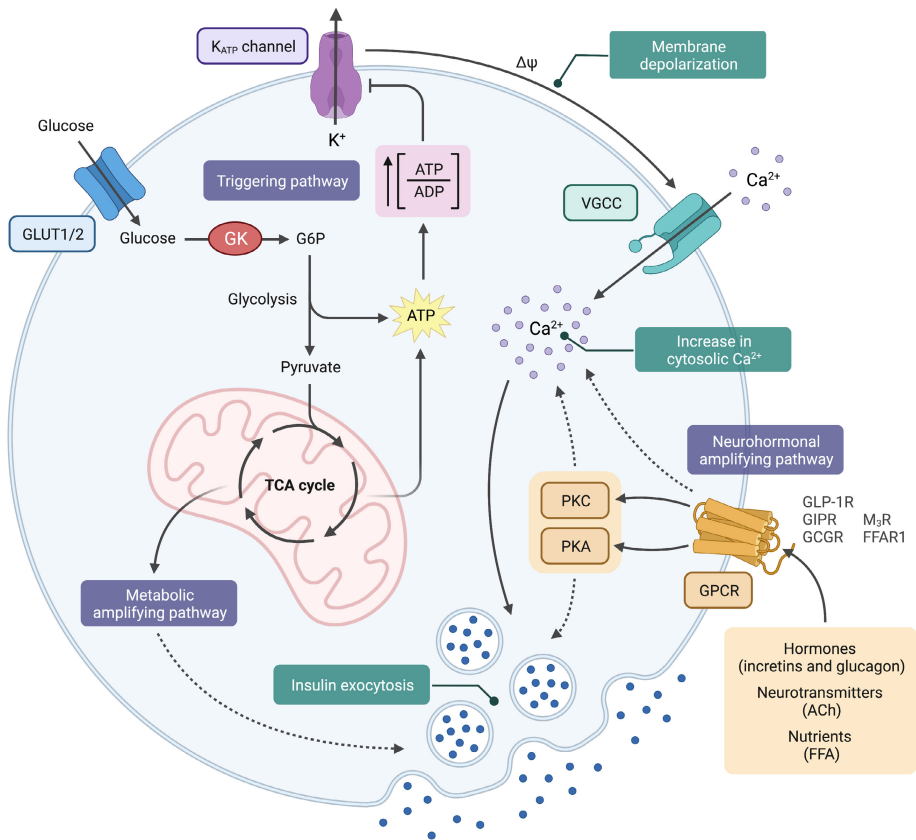


Figure 4. Main Pathways Governing Insulin Secretion in β -Cells. First, the triggering pathway is depicted, where glucose metabolism and ATP production lead to K_{ATP} channel closure, membrane depolarization, and Ca^{2+} influx, culminating in insulin exocytosis. Second, the metabolic amplifying pathway is showcased, underlining intracellular processes enhancing insulin secretion independently of K_{ATP} channel activity. Finally, the neurohormonal amplifying pathway is illustrated, underscoring the integral role of external signals such as hormones, neurotransmitters, and nutrients. These cues operate through G protein-coupled receptors (GPCRs) to further stimulate insulin release. ACh: acetylcholine; ADP: adenosine diphosphate; ATP: adenosine triphosphate; FFA: free-fatty acid; FFAR1: FFA receptor 1; G6P: glucose-6-phosphate; GCGR: glucagon receptor; GK: glucokinase; GIPR: GIP receptor; GLP-1R: GLP-1 receptor; GLUT, glucose transporter; K_{ATP} : ATP-sensitive potassium; M_3R : muscarinic 3 ACh receptor; PKA: protein kinase A; PKC: protein kinase C; TCA: tricarboxylic; VGCC: voltage-gated Ca^{2+} channel.

pivotal role in insulin secretion by acting as a glucose sensor in pancreatic β -cells. Importantly, glucose uptake is not rate limiting for GSIS, but rather the rate of β -cell glucose metabolism is controlled by GK, which determines the entry of glucose into the glycolytic pathway¹¹. GK catalyzes the phosphorylation of glucose to glucose-6-phosphate (G6P), a crucial initial step in glucose metabolism. Unlike other hexokinases, GK has a low affinity for glucose (K_m of approximately 8 mM), allowing it to respond effectively to changes in glucose concentrations. Thus, GK activity increases as blood glucose levels rise, leading to enhanced glycolysis^{73,74}. After being

phosphorylated, glucose is catabolized into pyruvate, which is then directed towards two pathways into the mitochondrial tricarboxylic acid (TCA) cycle in β -cells. One is through the oxidative enzyme pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-coenzyme A (acetyl-CoA). The other is through the anaplerotic enzyme pyruvate carboxylase (PC), which converts pyruvate to oxaloacetate. Approximately, an equal amount of pyruvate enters the TCA cycle through both pathways⁷⁵⁻⁷⁷. Although the conversion of pyruvate to lactate can occur in β -cells, it is limited due to the low levels of lactate dehydrogenase expression. This enzyme is one of several “disallowed genes” believed to be suppressed in β -cells, which helps to direct glucose to mitochondrial metabolism⁷⁸. Furthermore, β -cells exhibit significant activity in reducing equivalent shuttles, such as the glycerol phosphate and malate-aspartate shuttles. These shuttles convert cytosolic reduced nicotinamide adenine dinucleotide (NADH) to mitochondrial reduced flavin adenine dinucleotide (FADH) and NADH, which then fuels ATP production and thus contributes to the closure of K_{ATP} channels^{79,80}.

Although the K_{ATP} channel-dependent pathway is essential for activating the triggering phase of GSIS, amplifying K_{ATP} channel-independent pathways are active over the entire period of glucose stimulation and account for as much as 60-70% of insulin secreted in response to sustained glucose stimulation^{11,81,82}. The biochemical mechanisms of the amplifying phase of GSIS are complex and have been investigated exhaustively, and several models have emerged to explain it. For instance, glucose metabolism itself also contributes to the enhancement of insulin secretion by other stimuli that trigger the depolarization of β -cells through metabolic-amplifying pathways. These pathways are believed to be facilitated by mitochondrial-derived metabolic coupling factors, including TCA cycle intermediates, TCA cycle anaplerosis, guanosine triphosphate, or NADPH^{11,66}. Therefore, mitochondrial metabolism plays a crucial role in generating ATP, intersecting various metabolic pathways, and regulating the coupling factors for both triggering and metabolic-amplifying pathways, thus contributing to the typical biphasic insulin secretory response observed in β -cells^{11,83}.

Although glucose is the primary regulator of insulin secretion, circulating metabolites such as certain amino acids and lipids also impact this process²⁵. Accordingly, in β -cells, cationic amino acid transporters and sodium-coupled neutral amino acid transporters are highly expressed, and various mechanisms can trigger insulin secretion in response to amino acids^{84,85}. For instance, arginine, lysine, leucine, and glutamine can depolarize β -cells after their import, either directly by carrying a

positive charge⁸⁶ or via sodium co-transport⁸⁷. This depolarization leads to an influx of Ca^{2+} , subsequently triggering insulin secretion⁸⁸. Additionally, some amino acids, such as alanine, glutamate, and glutamine, fuel different components of the TCA cycle, leading to an increase in the ATP/adenosine diphosphate (ADP) ratio⁸⁹.

Insulin plays a crucial role in managing energy storing into triglycerides while concurrently suppressing lipolysis⁹⁰. Consequently, it is relevant for β -cells to exhibit responsiveness to circulating fatty acids to maintain a nutrient equilibrium. Thus, fatty acids of various chain lengths and saturation are subjected to intracellular fatty acid metabolism and acyl-CoA β -oxidation in the mitochondria, which in turn stimulates ATP production and insulin secretion via the aforementioned pathways⁹¹.

Furthermore, extracellular cues that interact with plasma membrane receptors can modulate the effects of glucose on insulin secretion, as these receptors can signal intracellularly and modulate β -cell function^{11,30,35}. A more detailed discussion on this topic will be presented in the next section.

1.1.4.2. Extracellular Signaling in the Regulation of Insulin Secretion

Extracellular signaling, an essential cellular communication mechanism, consists of three types: endocrine, paracrine, and autocrine, each critical for maintaining cellular balance. Endocrine signaling involves releasing signaling molecules into the bloodstream to impact distant cells, while paracrine signaling occurs between adjacent cells, and autocrine signaling takes place when a cell influences its own activity through signals it generates. These signaling forms, integral to regulating cellular responses to external stimuli, can operate in both positive and negative feedback loops⁹².

In the context of insulin secretion, the critical function of extracellular signaling mediated by hormones and neurotransmitters in the modulation of insulin secretion by β -cells has been recognized for several years. These signaling pathways not only provide an essential assessment of nutrient levels but also allow for precise coordination of insulin secretion with other physiological processes, ensuring proper regulation of glucose homeostasis^{25,35}.

One of these entities is **incretins**, specifically **glucose-dependent insulinotropic polypeptide (GIP)** and **glucagon-like peptide-1 (GLP-1)**, which are well-established hormones that play a crucial role in the regulation of glucose homeostasis and have garnered significant attention in the field of metabolic and cardiovascular

research. These hormones are primarily produced and secreted by enteroendocrine cells in the gastrointestinal tract, with GIP being synthesized by K cells and GLP-1 being produced by L cells^{93,94}. Both GLP-1 and GIP are synthesized as preprohormones and undergo a series of post-translational modifications (PTM) to generate their bioactive forms⁹⁴. Interestingly, GLP-1 shares a common precursor, proglucagon, with glucagon. Proglucagon is differentially processed by prohormone convertase enzymes in a tissue-specific manner, yielding glucagon in α -cells and GLP-1 in the intestinal L cells^{94,95}. Thus, while incretins and glucagon share a common precursor, their distinct roles in glucose homeostasis exemplify the intricacy and fine-tuning of hormonal regulation in metabolic processes.

The secretion of incretin hormones is regulated by multiple factors, mainly by nutrient ingestion, particularly carbohydrates and lipids, and also neural and hormonal signals. In response to food intake, GIP and GLP-1 are rapidly released into circulation, promoting their effects on various target organs, such as the stomach, pancreas, adipose tissue, and the CNS. Additionally, their secretion can be modulated by the gastric emptying rate, the presence of gastrointestinal peptides, and the action of various neurotransmitters^{93,96,97}.

Incretins exert their primary influence on β -cell function, thereby potentiating GSIS⁹³. Both GIP and GLP-1 bind to their respective G-protein-coupled receptors (GPCRs), GIPR and GLP-1R, on the surface of β -cell, leading to the activation of adenylyl cyclase (AC) and an increase in intracellular cyclic adenosine monophosphate (cAMP) levels⁹⁶. The subsequent activation of the protein kinase A (PKA) pathway induces Ca^{2+} release from intracellular stores and enhances insulin exocytosis⁹³. This potentiation of GSIS in insulin secretion caused by the consumption of oral nutrients is referred to as the **incretin effect**. This phenomenon is noteworthy because it results in a considerably higher insulin response compared to when glucose is administered intravenously (i.e., bypassing the gastrointestinal tract)³⁵. Furthermore, incretin hormones increase β -cell mass by promoting proliferation and differentiation, and inhibiting apoptosis, thereby preserving functional β -cell mass⁹⁴. Nonetheless, their potency is curtailed by enzymatic degradation. The primary regulator of this degradation is dipeptidyl peptidase-4 (DPP-4), which swiftly inactivates GLP-1 and GIP, thereby mitigating their metabolic impact. It is worth mentioning that GIP's potency in stimulating insulin secretion is diminished in individuals with T2D, whereas the insulinotropic action of GLP-1 remains largely preserved⁹³. This has led to the development of pharmacological agents targeting the GLP-1 receptor and

DPP-4 inhibitors, which have demonstrated significant benefits in the management of T2D and associated cardiovascular complications^{93,98}.

Furthermore, **neuronal regulation** of insulin secretion, particularly through the autonomic nervous system, is essential for maintaining glucose homeostasis and orchestrating the intricate interplay between various metabolic processes. The autonomic nervous system comprises both the sympathetic and parasympathetic branches, which modulate β -cell function by secreting neurotransmitters, such as acetylcholine, norepinephrine, and epinephrine, that bind to specific receptors on the β -cell^{99,100}.

Acetylcholine, released by parasympathetic neurons, acts on muscarinic 3 acetylcholine receptors (M_3R) on the β -cell, leading to the activation of phospholipase C (PLC), generation of inositol trisphosphate (IP_3), and subsequent release of intracellular Ca^{2+} stores, ultimately promoting insulin secretion^{35,101,102}. Conversely, the sympathetic nervous system exerts inhibitory effects on insulin secretion by releasing norepinephrine and epinephrine, which act on adrenergic receptors on the β -cell^{99,100}. **Epinephrine**, a catecholamine primarily produced by the adrenal medulla or sympathetic nerves, participates in the body's stress response and is released during perceived threats or danger¹⁰³. Epinephrine inhibits insulin secretion and stimulates glycogenolysis in the liver and muscles and gluconeogenesis in the liver, resulting in elevated blood glucose levels that provide an immediately accessible energy source during emergencies^{103,104}. The inhibitory effects of epinephrine on insulin secretion are primarily mediated through α_2 -adrenergic receptors, which reduce intracellular cAMP levels, contrasting with the mechanisms of incretin and muscarinic receptor activation^{102,105}.

While systemic signals are vital in regulating insulin secretion, the islet's structure establishes a unique microenvironment for local **intra-islet paracrine signals**. This additional layer of β -cell control is essential for coordinating insulin secretory responses. Factors secreted by various islet cells often interact with multiple receptors and cell types, enabling precise regulation of insulin secretion^{11,30}. For instance, the **glucagon receptor** (GCGR) expressed by β -cells can regulate and potentiate insulin secretion with α -cell-derived glucagon. Interestingly, GCGR and GLP-1R share high sequence homology, allowing their ligands to activate either receptor. Physiologically, this predominantly manifests as α -cell-derived glucagon signaling through both GLP-1R and GCGR on the β -cell^{35,106}.

Somatostatin is a hormone synthesized in various locations throughout the body. Its presence is prominent in the gastrointestinal tract and the anterior pituitary gland, where it plays an essential inhibitory role¹⁰⁷. Although pancreatic δ -cell-derived somatostatin only contributes to 5-10% of systemic circulating levels, its primary function is believed to be paracrine regulation^{35,108}. Spanning a wide physiological glucose range, δ -cells initiate secretion at glucose concentrations as low as 3 mM and progressively increase linearly up to 20 mM. This extensive activity range directly influences α - and β -cell inhibition^{35,107}. Somatostatin receptor signaling not only inhibits hormone secretion by reducing AC activity but also simultaneously activates G-protein-gated inwardly rectifying K⁺ channels, counteracting glucose-mediated membrane depolarization and inactivating VGCCs³⁵. Under high glucose conditions, somatostatin release provides inhibitory feedback that does not entirely suppress β -cells but offers tonic inhibition. This configuration likely prevents excessive insulin release, thereby avoiding insulin-induced hypoglycemia¹⁰⁹. Another paracrine regulator of β -cell function is ϵ -cell-derived ghrelin during fasting conditions, which can also inhibit insulin secretion³⁵. While ghrelin's impact on β -cell function is appreciable, it is only one of the numerous intra-islet and systemic factors that orchestrate the complex process of insulin secretion^{11,35}.

Over the last two decades, extensive research has uncovered that **autocrine signaling** mediated by insulin itself plays a critical role in β -cell proliferation, survival, and function^{110–112}. Remarkably, mice lacking the insulin receptor in β -cells displayed defects in insulin secretion in response to glucose and progressive impairment of glucose tolerance¹¹¹. Moreover, insulin signaling is crucial for postnatal β -cell growth and maintaining β -cell mass¹¹³. Continuous exposure of β -cells to high insulin levels can result in desensitization of the signaling cascade, which may result in a lack of response¹¹⁰. However, the acute effects of insulin on stimulus-secretion coupling remain less clear¹¹⁴. Moreover, a recent study discovering the role of a novel protein in β -cells, the inhibitor of insulin receptor or inceptor, reinforces the long-held notion that insulin signaling plays a crucial role in regulating β -cell biology¹¹⁵.

Moreover, β -cells within an islet synchronize their electrical and Ca²⁺ responses through **gap junctional coupling**, providing a robust insulin secretory response²⁵. Recent studies have demonstrated that, within islets, β -cells may take on different roles, some acting as pacemaker or hub cells. These specialized β -cells are characterized by their ability to generate rhythmic electrical activity, leading to synchronized Ca²⁺ oscillations and coordinated insulin release among neighboring β -cells through

gap junctions^{119,120}. However, the precise mechanisms underlying the formation and regulation of hub cells within islets remain an active area of research²⁵.

Nutrient sensing by extracellular receptors in β -cells is a rapidly developing area of interest, as it provides an essential regulatory mechanism that ensures proper adaptation to fluctuating nutrient levels. By detecting changes in nutrient concentrations, including **amino acids** and **fatty acids**, and subsequently modulating β -cell function, these receptors maintain glucose homeostasis and coordinate insulin secretion with other physiological processes. Among these receptors, the most extensively studied is the **free-fatty acid receptor 1 (FFAR1)**, a GPCR highly expressed in β -cells. Activation of FFAR1 enhances GSIS via protein kinase C (PKC) and Ca^{2+} signaling pathways. Additionally, FFAR1 signaling initiates diacylglycerol (DAG)-mediated activation of protein kinase D1, which facilitates the remodeling of cortical F-actin and the recruitment of secretory granules to the readily releasable pool¹¹⁶.

Overall, extracellular signaling cues, including endocrine, paracrine, and autocrine mechanisms, are all essential components of insulin secretion regulation. In particular, nutrient sensing is emerging as a crucial aspect for proper glucose homeostasis and regulation of postprandial states, as it may enable β -cells to adapt their insulin secretion in response to fluctuations in nutrient levels.

1.1.5. Insulin Actions in Metabolism Regulation

Having explored the complexities of insulin release in β -cells in the previous section, it is now essential to broaden the understanding of insulin action beyond secretion. This will enable a more comprehensive view of insulin's role in glucose homeostasis and its influence on various physiological processes. A key element in this context is the insulin receptor (IR), which serves as the primary mediator of insulin signaling. By linking the knowledge of insulin secretion with the downstream effects of IR, a deeper insight into the intricate regulatory mechanisms governing glucose metabolism and energy balance can be attained.

From a structural point of view, the IR is a heterotetrameric protein composed of two extracellular α -subunits and two transmembrane β -subunits linked by disulfide bonds¹¹⁷. Upon insulin binding, the IR initiates two primary signaling cascades: the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the mitogen-activated protein kinase (MAPK) pathway. The **PI3K-Akt pathway**, critical for mediating insulin's metabolic actions like glucose uptake and lipogenesis, is triggered via IR phosphorylation of insulin receptor substrate (IRS) proteins. The **Ras/MAPK pathway**, cru-

cial for cell growth and survival, is activated through the binding of growth factor receptor-bound protein 2 to IRS proteins. Hence, the IR crucially regulates glucose homeostasis, cellular growth, and differentiation across various organs, highlighting the importance of insulin signaling in maintaining overall metabolic balance and health¹¹⁸ (**Figure 5**).

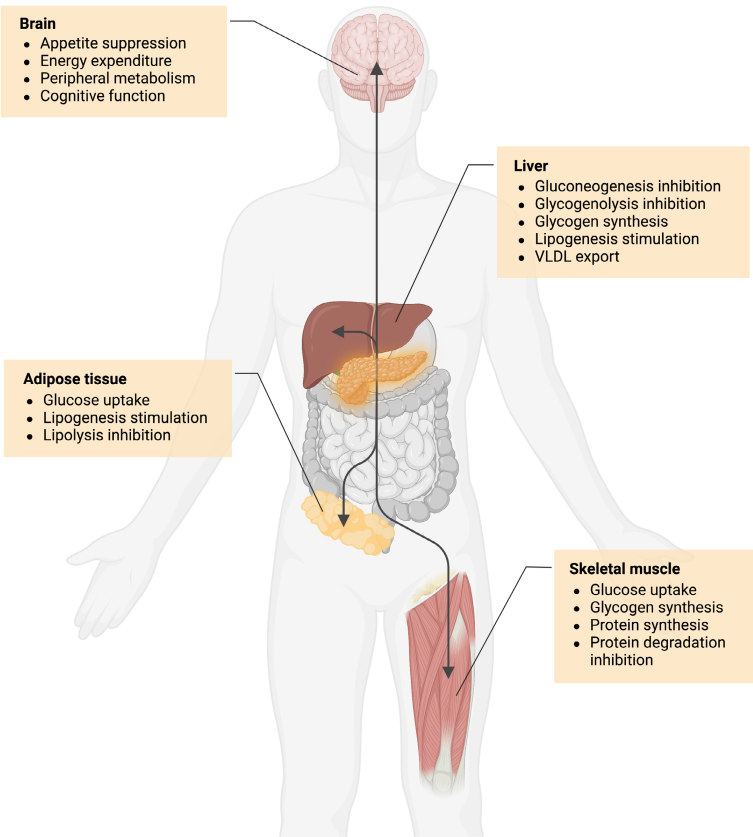


Figure 5. Insight into Insulin Actions. Insulin executes diverse functions across an array of target tissues. In peripheral tissues, insulin’s influence is ubiquitous, but it predominantly asserts substantial impacts on metabolic tissues, namely adipose tissue, liver, and skeletal muscle. Moreover, insulin navigates the blood-brain barrier, wielding its regulatory capacities within the brain directly while also initiating indirect signaling pathways communicating back to the peripheral organs. VLDL: very low-density lipoprotein.

In **skeletal muscle**, insulin stimulates glucose uptake by promoting the translocation of GLUT4 to the plasma membrane, which increases glucose transport into myocytes. In addition to this, IR signaling also enhances glycogen synthesis by activating glycogen synthase and inhibiting glycogen phosphorylase. Furthermore, it

stimulates protein synthesis and inhibits protein degradation, thereby promoting muscle growth and maintenance^{10,119}.

In **adipose tissue (AT)**, insulin plays a crucial role in storing and utilizing energy. It induces GLUT4 translocation to the plasma membrane and stimulates lipogenesis by upregulating enzymes involved in fatty acid and triglyceride synthesis. Simultaneously, insulin inhibits lipolysis, thereby reducing the release of free fatty acids into the circulation. This dual role in AT is essential in maintaining energy balance and preventing lipotoxicity in other tissues^{10,118,120}.

While the **liver** is less dependent on insulin for glucose uptake than skeletal muscle and adipose tissue, IR signaling exerts essential control over hepatic glucose production and lipid metabolism¹²¹. Primarily, insulin suppresses hepatic glucose production by inhibiting glycogenolysis and gluconeogenesis, and it encourages glucose storage by promoting glycogenesis^{10,122}. This careful balance supports postprandial glucose homeostasis. Regarding lipid metabolism, insulin stimulates lipogenesis, thereby aiding the synthesis and storage of fatty acids during periods of nutrient surplus. Furthermore, insulin promotes the assembly and secretion of very low-density lipoproteins (VLDLs), which carry these synthesized lipids from the liver to peripheral tissues. This process is vital in the redistribution of lipids throughout the body, underscoring the multi-faceted role of insulin in regulating metabolic homeostasis^{121,123}.

The insulin signaling pathway also plays a critical role in mediating energy expenditure and food behavior in the **CNS**. IR is expressed in various brain regions, including the hypothalamus, which regulates energy balance and food intake. Accordingly, insulin regulates the activity of neurons that either reduce food intake or increase energy expenditure, depending on the availability of nutrients in the body¹²⁴. Additionally, insulin signaling in the brain also exerts an indirect influence on peripheral tissue metabolism, fostering a comprehensive, system-wide metabolic regulation¹²⁵. Insulin also affects the activity of brain regions involved in reward and motivation, such as the mesolimbic dopamine system, where insulin signaling can suppress food-seeking behavior¹²⁶.

Alterations in IR signaling, arising from genetic mutations or reduced insulin sensitivity, can lead to insulin resistance, a key pathological feature of T2D¹⁰. In addition to its role in T2D, dysregulation of IR signaling has been implicated in various other pathological conditions, such as cancer, cardiovascular disease, and neurodegenerative disorders. Hyperinsulinemia, often observed in insulin-resistant states,

can promote tumorigenesis by enhancing cell proliferation and survival through activating the IR or also insulin-like growth factor 1 receptor signaling pathways¹²⁷. Moreover, the relationship between altered insulin signaling and neurological or psychiatric disorders is an area of growing research interest. This is particularly relevant to neurodegenerative disorders, such as Alzheimer's disease, as insulin and the IR are involved in regulating synaptic function, learning, and memory^{128,129}. Furthermore, dysregulation of insulin signaling may contribute to developing or exacerbating various neuropsychiatric conditions, including depression, anxiety, and schizophrenia¹³⁰⁻¹³².

1.2. Diabetes Mellitus

Diabetes mellitus is a group of metabolic disorders characterized by **hyperglycemia**, resulting from defects in insulin secretion, insulin action, or both¹³³. The chronic hyperglycemia associated with diabetes leads to long-term complications affecting various organs, including the eyes, kidneys, nerves, and the cardiovascular system¹³⁴. There are three major types of diabetes: type 1 diabetes (T1D), **type 2 diabetes (T2D)**, and gestational diabetes mellitus (GDM). Other less common types include monogenic diabetes and diabetes due to specific conditions or syndromes¹³³.

T1D, previously known as insulin-dependent or juvenile-onset diabetes, is an autoimmune disorder characterized by the destruction of β -cells, leading to an absolute insulin deficiency¹³⁵. T1D accounts for approximately 5-10% of all diabetes cases and usually occurs in children and young adults¹³³. Although genetic predisposition plays a significant role in the development of T1D, environmental factors, such as viral infections and early exposure to certain dietary components, have also been implicated¹³⁶. Treatment for T1D requires lifelong insulin therapy, along with continued blood glucose monitoring and a healthy lifestyle¹³⁵.

T2D, formerly known as non-insulin-dependent or adult-onset diabetes, is the most common form of diabetes, accounting for about 90-95% of all cases¹³³. T2D is characterized by insulin resistance, a condition in which cells fail to respond appropriately to insulin, and a relative insulin deficiency¹³⁷. The pathophysiology of T2D is complex and involves genetic and environmental factors, such as obesity, a sedentary lifestyle, and an unhealthy diet, which contribute to insulin resistance and β -cell dysfunction¹³⁸.

GDM is a form of diabetes that occurs during pregnancy, affecting approximately 13% of pregnancies worldwide¹⁵. GDM is characterized by glucose intolerance that is first identified during pregnancy and usually resolves after delivery¹³³. However, women with a history of GDM are at an increased risk of developing T2D later in life, and their offspring also have a higher risk of obesity and T2D¹³⁹. A meta-analysis revealed that women with previous GDM have almost ten times higher risk of developing T2D than healthy individuals¹⁴⁰, emphasizing the need for effective prevention strategies during pregnancy and postpartum. Management of GDM includes lifestyle modifications, blood glucose monitoring, and insulin therapy, if required, to prevent complications for both the mother and the newborn¹³³.

Monogenic diabetes is a rare form of diabetes caused by mutations in a single gene, accounting for 1-2% of all diabetes cases¹⁵. Monogenic diabetes includes maturity-onset diabetes of the young and neonatal diabetes mellitus, both characterized by impaired β -cell function¹⁴¹. The specific treatment for monogenic diabetes depends on the underlying genetic defect, and in some cases, patients may be able to discontinue insulin therapy and switch to oral medications¹⁴¹.

In summary, diabetes mellitus is a group of metabolic disorders with various etiologies and clinical manifestations. Among them, T2D represents the most common form, accounting for an overwhelming majority of the global diabetes burden. Given the rising prevalence and associated complications of T2D, there is an urgent need for effective prevention and management strategies. In the following sections, a comprehensive overview of the epidemiology, complex pathophysiology, risk factors, and current therapeutic approaches for T2D will be provided.

1.2.1 The Escalating Global Prevalence and Impact of Type 2 Diabetes

The **increasing prevalence** of T2D is intimately connected to the growing incidence of obesity, stemming from multiple factors such as economic growth, urbanization, unhealthy dietary habits, and sedentary lifestyles¹⁴². The disparity between natural environments and Westernized societies can substantially explain this surge. In nature, energy sources are often limited and unpredictable, necessitating the evolution of mechanisms for efficient energy storage and utilization, as well as the adaptation of organisms to capitalize on food encounters. Conversely, contemporary technologically advanced societies experience a diminished caloric demand while being inundated with readily available, alluring, and calorie-dense products^{143,144}. This continuous transformation has significantly contributed to the pervasive prevalence of both obesity and T2D^{15,144}.

T2D is a significant **public health problem worldwide**, with an estimated 500 million adults living with the condition globally^{15,133}. By 2045, this number is projected to increase to approximately 740 million¹⁵, with a greater proportion of individuals with T2D residing in low- to middle-income countries in comparison to high-income ones due to the gradual increase in Westernization¹⁴⁵.

Undiagnosed diabetes affects about 45% of the global population, with Africa having the highest proportion of undiagnosed cases¹⁵. Additionally, approximately 352 million people have impaired glucose tolerance or fasting glucose, which can progress

to T2D at a rate of 5-10% per year¹⁴⁶. Although Africa has the lowest prevalence of T2D, it is expected to experience the highest increase in the next 25 years¹⁵. Environmental factors and natural and man-made disasters also exacerbate disparities in the distribution and burden of the disease¹³⁸.

The **economic burden** of T2D encompasses both direct and indirect costs. Direct costs refer to the medical expenses incurred in diagnosing, treating, and managing T2D, such as hospitalizations, medications, outpatient care, and emergency room visits. According to the International Diabetes Federation (IDF), hospitalizations related to T2D represent a considerable portion of healthcare expenses worldwide, indicating the pressing need for effective prevention and management strategies. In 2021, the IDF estimated the total cost of diabetes to be \$327 billion¹⁵, with inpatient care comprising 43% of the total expenditure in the United States¹⁴⁷. In Spain, the approximate total expenditure on diabetes-related healthcare is \$15.5 billion¹⁵, underscoring the substantial financial impact of this disease for the public healthcare system. The rising cost of insulin and other diabetes medications has been a significant driver of healthcare expenses related to T2D¹⁴⁸. Moreover, the ongoing management of T2D often requires regular doctor visits, laboratory tests, and preventive care, which, along with complications such as hypoglycemia or hyperglycemia that can lead to emergency room visits, accounted for 18% of the total cost of diabetes in the United States¹⁴⁷.

Indirect costs associated with T2D encompass productivity loss and disability¹⁴⁷, primarily stemming from **long-term complications** associated with T2D, such as cardiovascular disease, kidney failure, and vision impairment. The World Health Organization (WHO) reported that diabetes was the eighth leading cause of disability globally. Additionally, the WHO has identified diabetes as the ninth leading cause of death worldwide¹⁴⁹. Accordingly, T2D is a major contributor to premature mortality on a global scale, with the IDF estimating that 6.7 million diabetes-related deaths occurred in 2021, the majority of which were attributable to T2D¹⁵.

The global incidence and prevalence of obesity and T2D among individuals under the age of 40 are on the rise, with rates varying depending on factors such as age, sex, ethnicity, and geography. **Early-onset T2D** presents a more aggressive metabolic phenotype associated with increased insulin resistance, early β -cell failure, and an earlier onset of complications^{150–152}. In fact, nearly 60% of individuals with this condition experience at least one complication by early adulthood¹⁵³. Unfortunately, there are limited treatment options available for young people with T2D, as

there is little long-term safety data to support the use of glucose-lowering therapies in these populations¹⁵¹. To address these concerns, novel treatment approaches and innovative study designs are needed, as only a few trials have specifically evaluated the safety and efficacy of glucose-lowering therapies in individuals under 40^{151,154}.

Overall, these dire statistics emphasize the critical need for continued research and development of new therapies to combat T2D. The current trend of increasing T2D rates, poor glycemic control, and emerging early-onset T2D in the young requires a reversal in this trajectory to improve outcomes for individuals and society at large. By expanding our understanding of the underlying pathophysiology and risk factors associated with T2D, novel interventions can be developed to improve prevention, diagnosis, and management. Such advancements can potentially reduce the overall burden of T2D on individuals and society, improve quality of life, and ultimately save lives.

1.2.2. The Pathophysiology of Type 2 Diabetes

T2D and obesity are complex and interrelated diseases with overlapping pathological mechanisms, including ectopic adiposity, insulin resistance, inflammation, and β -cell dysfunction¹⁵⁵. However, the underlying pathophysiological mechanisms that explain how overnutrition and chronic adipose tissue accumulation predispose to insulin resistance, β -cell dysfunction, and ultimately T2D remain unclear. Notably, not all individuals who are overweight or obese will develop T2D, and conversely, some people with a healthy weight can be diagnosed with the disease, indicating that other factors may play a role in its pathogenesis¹³⁸. Recent advances in understanding the biological mechanisms of obesity and T2D suggest a significant genetic influence in disease susceptibility, with heritability estimated at 30-70%^{156,157}.

The **development of T2D** is characterized by **insulin resistance** leading to elevated glucose levels, which in turn induces β -cells to ramp up insulin production, an adaptative response that unfortunately contributes to their eventual decay (**Figure 6**). Insulin resistance marks the first stage in T2D development, leading to increased β -cell stimulation and insulin secretion to maintain normal glucose levels¹⁵⁸, which is known as **compensatory hyperinsulinemia**. Remarkably, it has been found that, at the time of diagnosis, up to 80% of β -cell function may already be lost^{159,160}, with further loss of 4-5% per year^{161,162} and becoming irreversible after a T2D duration of 10 years¹⁶³. While most people are able to maintain normoglycemia with a compensatory increase in insulin secretion, individuals susceptible to developing T2D may

eventually experience β -cell failure, leading to glucose intolerance, fasting hyperglycemia, and ultimately overt diabetes¹⁴. However, the rate of progression to T2D can vary among individuals¹⁶⁴, and the restoration of substantial functional β -cell mass has been observed with adequate glycemic control or disease remission^{159,160}. Consequently, a **β -cell-centric model** that recognizes abnormal β -cell function as a primary defect of T2D has been proposed¹⁶⁵.

1.2.2.1. Prediabetes: The Latent Prelude of Type 2 Diabetes

Prediabetes is an intermediate metabolic state and critical stage in the development of T2D, which is often overlooked due to its asymptomatic nature. It is characterized by elevated blood glucose levels higher than normal but not elevated enough to be diagnosed as T2D¹⁴⁶. This condition is associated with an increased risk of developing T2D, cardiovascular disease, and other health complications¹³³.

Two standard measures are used to define prediabetes: **impaired fasting glucose (IFG)** and **impaired glucose tolerance (IGT)**. Elevated fasting plasma glucose levels characterize IFG, while IGT refers to elevated blood glucose levels after a glucose challenge, typically during an oral glucose tolerance test¹³³. Both IFG and IGT are indicative of abnormalities in glucose metabolism and insulin resistance and can be used to identify individuals at high risk of developing T2D^{133,146}.

The intricate trajectory from normoglycemia through prediabetes to eventual T2D is a multifaceted process orchestrated by numerous factors, encompassing genetic predisposition, environmental interactions, and lifestyle determinants¹³⁸. A pivotal element in this development is insulin resistance, yet the specific mechanisms provoking an augmentation in β -cell activity, culminating in their dysfunction within the prediabetic state, remain inadequately elucidated¹⁶⁶.

Research has shown that early intervention, such as lifestyle modifications and pharmacological treatments, can prevent or delay the onset of T2D in individuals with prediabetes¹⁴⁶. Therefore, recognizing and addressing prediabetes before it progresses to T2D is crucial for reducing the impact of diabetes and its associated health complications.

1.2.2.2. Insulin Resistance

In T2D, alterations in the insulin signaling cascade have been observed at multiple levels, encompassing reduced IR expression, impaired IR autophosphorylation, di-

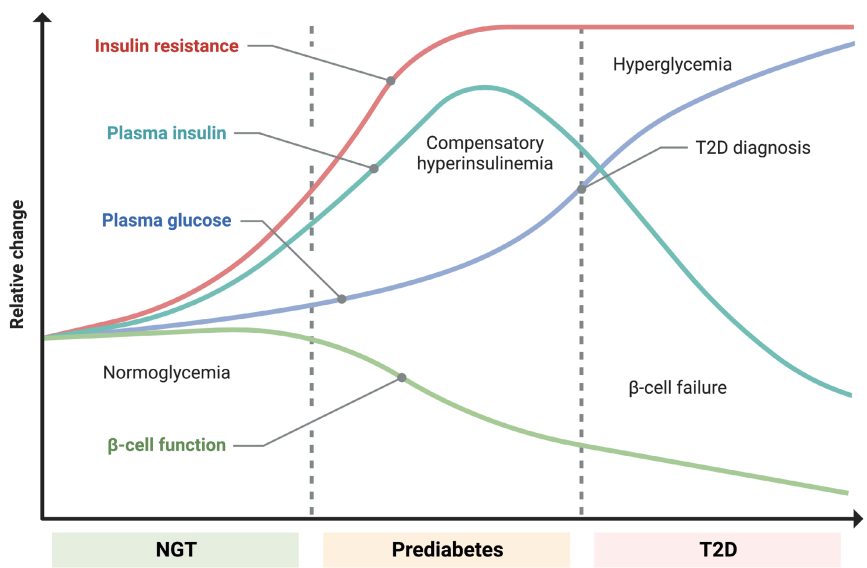


Figure 6. Chronological Progression of Type 2 Diabetes. This graph delineates the relative evolution of key metrics –glucose levels, plasma insulin, and insulin resistance– throughout the transition from normal glucose tolerance (NGT) to prediabetes and, ultimately, to established type 2 diabetes (T2D). In the prediabetes phase, compensatory hyperinsulinemia is emphasized, illustrating the β -cell's counteractive response to burgeoning insulin resistance. As the disease advances, the graph accentuates the decline in β -cell function, a primary catalyst in T2D progression.

minished IRS protein expression and function, and impaired downstream signaling mediators, which can be provoked by genetic and environmental factors^{121,167}. elevated levels of pro-inflammatory signals are closely associated with the development and progression of metabolic disorders such as obesity and T2D. These conditions often stem from chronic low-grade inflammation¹⁶⁸ and lipotoxicity⁹⁰, both of which can interfere with insulin signaling and exacerbate insulin resistance. It is important to note that the precise molecular and cellular mechanisms underlying insulin resistance can vary across the different peripheral tissues¹⁰.

In the **liver**, insulin resistance can lead to increased gluconeogenesis and impaired glycogen synthesis¹⁰. Chronic inflammation, activation of c-Jun N-terminal kinases (JNKs), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathways contribute to the dysregulation of insulin signaling, leading to an imbalance in glucose production and storage¹⁶⁸. The accumulation of lipids, particularly DAG, has also been implicated in hepatic insulin resistance, as it activates PKC isoforms, further impairing insulin signaling¹²¹.

Skeletal muscle is responsible for a significant portion of insulin-stimulated glucose uptake¹⁰. In this tissue, insulin resistance has been associated with defects in

GLUT4 trafficking and impaired activation of the PI3K-Akt signaling pathway^{10,121}. Inflammation, oxidative stress, and lipid accumulation also contribute to insulin resistance in skeletal muscle^{121,168}.

In **AT**, insulin resistance is linked to dysregulated lipolysis, inflammation, and altered adipokine secretion¹⁰. AT inflammation, mediated by cytokines like tumor necrosis factor- α (TNF- α) and interleukin 1 beta (IL-1 β), can impair insulin signaling and contribute to systemic insulin resistance^{168,169}. The ectopic accumulation of lipids in non-adipose tissues, such as the liver and muscle, also contributes to insulin resistance¹²¹.

T2D pathophysiology was once thought to involve only the triumvirate of muscle, liver, and β -cell interplay. However, it is currently acknowledged that dysregulation of incretins, lipolysis, hyperglucagonemia, increased glucose reabsorption in the kidney, and central effects, such as appetite dysregulation, also play critical roles in T2D development¹⁷⁰. This interplay has been further expanded to include defects in multiple interlocking pathways, with β -cell dysfunction as the common denominator connecting factor. Thus, **β -cell impairment** is considered the **central hallmark** of T2D pathophysiology¹³⁸.

1.2.2.3. β -Cell Failure in Type 2 Diabetes

Alterations in islet morphology and function are prominent characteristics of T2D progression, predominantly driven by changes in β -cell function and mass as a response to metabolic stress^{25,171}. These adjustments encompass changes in islet size and cell composition and coincide with three interconnected pathophysiological mechanisms at the heart of β -cell failure. These mechanisms, which are not mutually exclusive, include β -cell exhaustion, reduction in β -cell mass, and compromised β -cell identity⁵⁸, all crucially contributing to the progression and severity of the disease (**Figure 7**).

The first mechanism is **β -cell exhaustion** caused by **oxidative stress** as a result of altered glucose metabolism^{159,172,173}. Several factors may contribute to this phenomenon, including **inflammatory**, **endoplasmic reticulum (ER)**, and **metabolic stress**¹⁷⁴. An increased metabolic load arising from insulin resistance, hyperglycemia, or hyperlipidemia may place β -cells under severe ER stress, leading to β -cell exhaustion, where β -cells fail to secrete insulin despite being structurally normal^{175,176}. Notably, the ER in β -cells plays a critical role in insulin biosynthesis and is responsible for about half of the total protein these cells produce. Proinsulin production

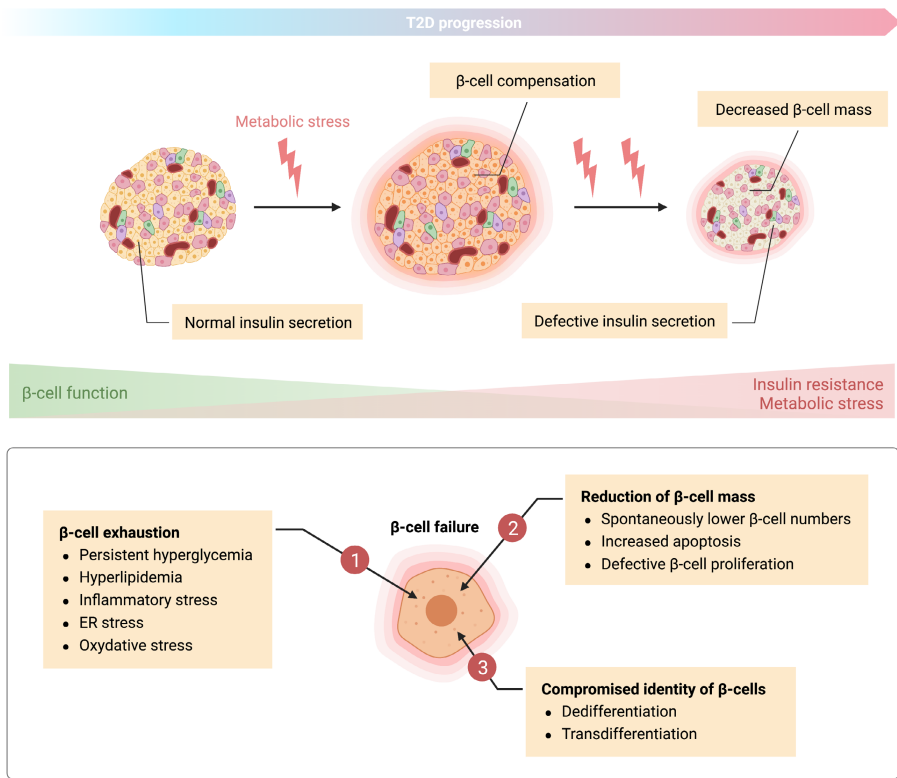


Figure 7. β-Cell Failure in Type 2 Diabetes. Alterations in islets, primarily steered by β-cells, during the progression of type 2 diabetes (T2D) are depicted, which significantly impact both insulin secretion and overall β-cell mass. The principal mechanisms leading to β-cell failure, namely β-cell exhaustion, reduction in β-cell mass, and compromised β-cell identity, are displayed. ER: endoplasmic reticulum.

places a significant burden on the ER, especially under compensatory hyperinsulinemia. Failure to regulate proteostasis can cause ER stress, triggering the **unfolded protein response (UPR)**, a signaling pathway that can affect the survival and proliferation of β-cells. While successful UPR can promote cell survival and elevated β-cell numbers by increasing protein folding capacity, in some cases, it can result in collateral damage and contribute to the pathogenesis of diabetes^{159,177}.

Moreover, **hyperglycemia**, **hyperlipidemia**, and **inflammation** can induce the generation of **reactive oxygen species (ROS)** in β-cells, causing damage to them. While ROS has a role in promoting insulin secretion under normal conditions, studies have shown that ROS production becomes excessive under pathological conditions, resulting in oxidative stress. The mitochondrial electron transport chain is the primary contributor to ROS production, which becomes overloaded in hyperglycemic and hyperlipidemic conditions. Although β-cells have antioxidant defense mechanisms such as catalase and glutathione peroxidase 1, their lower expression

levels suggest higher susceptibility to ROS damage¹⁷⁸. Reversing β -cell exhaustion may be achievable by restoring normal glucose and lipid levels^{179,180}. Calorie restriction, for instance, is one potential method to accomplish this^{181,182}. In fact, a study demonstrated that β -cell function could improve as early as four weeks after initiating a very low-calorie diet¹⁸¹.

The second mechanism is the **reduction of β -cell mass**, which can result from genetic or environmental factors. Autopsy studies have shown that β -cell mass can decrease by up to 60% in people with T2D compared to healthy individuals with the same body mass index (BMI)^{183,184}. β -cells have a long lifespan with **limited potential for neogenesis or replication**, and multiple observations suggested that the human β -cell mass is established within the first 2-3 decades of life, after which β -cells age with the body¹⁸⁵⁻¹⁸⁷. Moreover, it is possible that individuals with **spontaneously low β -cell mass** may lack the capability to enhance β -cell capacity when faced with increased demand, potentially putting them at a higher risk of developing T2D^{188,189}. In T2D islets, the total α -cell mass remains relatively unchanged¹⁹⁰, while the reduction in β -cell mass is less pronounced in recent-onset cases and gradually becomes more significant with the progression of the disease, which, unlike the rapid evolution observed in T1D, the loss of β -cells occurs over a much larger extended period^{184,191}. Despite increased apoptosis being a rare event in T2D β -cells, detecting this phenomenon *in vivo* is challenging as apoptotic cells are rapidly removed by adjacent cells¹⁹². Moreover, β -cell apoptosis is believed to occur most likely during the persistent hyperglycemia period around the clinical onset of T2D¹⁹³. Notwithstanding the aforementioned, β -cell dysfunction is a critical factor in T2D pathogenesis and can occur independently of β -cell loss, with an estimated reduction of up to 80% in β -cell function at T2D onset^{194,195}.

The third mechanism involves the **compromised identity of β -cells**, resulting from dedifferentiation or transdifferentiation into other cell types. **Dedifferentiation**, characterized by hormone-negative endocrine islet cells, is prevalent in T2D but varies significantly^{196,197}. **Transdifferentiation** is a form of cellular reprogramming in which a differentiated cell is converted into a different type of cell, bypassing the pluripotent state. A noteworthy phenomenon related to transdifferentiation in T2D is the occurrence of cells within the islets that simultaneously express glucagon and insulin. Additionally, cells simultaneously containing insulin along with amylase-loaded acinar granules are observed. Such occurrences are less frequently noted in non-diabetic islets¹⁹⁸⁻²⁰⁰. These events imply a loss of specificity in hormone expression within islet cells, potentially compromising their identity and function.

Particularly in β -cells, the expression of **canonical identity markers** is important for understanding the pathophysiology of T2D^{25,197}. For instance, a preclinical study in mice with forkhead box protein O1 (FOXO1)-deficient β -cells suggested that a high metabolic load can cause the loss of β -cell gene expression, leading to dedifferentiation and reprogramming of β -cells that results in a phenotype similar to other islet cell types, resulting in loss of β -cell function without cell death²⁰¹. However, subsequent studies have shown that dedifferentiated β -cells may revert to their original identity when exposed to normal glucose levels^{202,203}.

1.2.2.4. Approaches to Assess In Vivo Human β -Cell Function

β -cells secrete **C-peptide** and insulin in equimolar amounts into the portal vein⁶⁵. While insulin undergoes hepatic degradation and has a short half-life of 3-5 minutes, C-peptide is cleared entirely in peripheral tissues at a relatively constant rate and has a longer half-life of approximately 35 minutes. Insulin assays cannot distinguish between endogenous and exogenous insulin in individuals who receive insulin therapy. However, due to the differential kinetics of C-peptide, peripheral plasma C-peptide concentrations can be used to estimate insulin secretion accurately^{204,205}. Additionally, the **homeostasis model assessment of β -cell function (HOMA-B)** is an indirect measure of β -cell function calculated using fasting plasma glucose and insulin levels; however, it provides limited information on daily fluctuations in glucose^{206,207}.

Currently, measuring **glycated hemoglobin (HbA1c)** is the most common method for assessing glycemic control in individuals with T2D. However, it only provides an indication of the average blood glucose level for the previous 2-3 months and does not consider β -cell reserve and function, which are essential indicators of disease status and the need for additional or alternative treatments²⁰⁸. Measuring β -cell function is critical in people with high Hb1Ac levels or long T2D duration, as it can help guide appropriate treatment choices. Knowing β -cell function can also help avoid the use of unnecessary diabetes medications that may not achieve glycemic control in people with marked hyperglycemia¹⁵⁹.

Tests to assess glycemic control after a nutrient challenge, such as the **oral glucose tolerance test (OGTT)** and the meal tolerance test (MTT), involve measuring glucose, C-peptide, glucagon, and insulin concentrations and are more direct measures of β -cell function compared to the aforementioned methods. OGTT is a well-established diagnostic procedure, is easily performed in numerous clinical settings, and

can be effectively utilized in large-scale clinical trials²⁰⁹. As an oral test, it activates the incretin effect and has specific reference values depending on blood glucose levels, where normal levels are below 139 mg·dl⁻¹, prediabetes levels are 140 to 199 mg·dl⁻¹, and diabetes levels are above 200 mg·dl⁻¹. MTT is more challenging to standardize but is a direct measure of β -cell function after nutrient absorption from the gut since it includes lipids and amino acids, which influence β -cell function as well²⁰⁷.

However, identifying an ideal approach for assessing insulin resistance or secretion remains elusive in both clinical practice and extensive medical research. Though several practical methods have been suggested for use in large-scale clinical trials, they have faced scrutiny due to shortcomings when examining individuals with diabetes, diverse etiologies of glucose imbalances, or varied racial and ethnic backgrounds^{210,211}. Additionally, certain methods only capture a fraction of the intricate associations that govern glucose-insulin equilibrium²¹¹. The scientific community widely agrees that the **euglycemic-hyperinsulinemic clamp** allows for insulin resistance measurement, while the **hyperglycemic clamp** facilitates the evaluation of insulin secretion. Nonetheless, these procedures necessitate skilled professionals and are resource-intensive, confining their usage primarily to specialized research facilities and limiting the number of test subjects they can accommodate²⁰⁹.

In comparison to these intricate techniques, the OGTT, when combined with isoglycemic intravenous glucose infusion (IIGI) and mathematical modeling, provides a more straightforward approach for evaluating the relative contribution of the incretin effect on insulin secretion potentiation^{212–214}. The IIGI technique entails replicating the OGTT glucose profile through adjustable intravenous glucose administration. Thus, by circumventing the gastrointestinal tract and reproducing the glucose profile observed during the OGTT, the incretin effect can be estimated using mathematical modeling that capitalizes on the data gathered from both tests^{212,213}.

A significant advantage of **mathematical modeling** in assessing β -cell function lies in its capacity to offer insights into intricate physiological systems and processes that would otherwise be challenging to observe or quantify. These models function by utilizing equations to represent physiological processes and systems, formulated based on experimental observations, data from clinical studies, and established principles of physiology and biochemistry²¹⁵.

In general, mathematical models involve three key components: input variables, output variables, and the mathematical relationships between them. The input

variables represent the factors influencing the system, such as blood glucose levels, while the output variables represent the system's response, such as insulin secretion. In the context of β -cell function assessment, mathematical models often rely on differential equations to describe the dynamic processes occurring within the β -cells. These equations capture the interplay between various cellular components, signaling pathways, and molecular interactions contributing to insulin secretion. For example, models may consider the role of Ca^{2+} signaling pathways, K_{ATP} channels, glucose transporters, and incretin hormones, among other factors^{215,216}. By analyzing the behavior of these models under different conditions, a deeper understanding of the factors that influence β -cell function and insulin secretion can be achieved. Moreover, mathematical models can be used to estimate the integrative parameters that characterize the individual β -cell function, which can be of great clinical value in research and personalized medicine²¹⁵.

1.2.2.5. Diabetes Complications

Diabetes mellitus is associated with a plethora of complications affecting multiple organ systems. These complications, which can be generally classified into microvascular and macrovascular, profoundly contribute to increased morbidity and mortality among individuals with diabetes¹³⁸. Individuals with T2D who have long-standing diabetes, suboptimal glycemic control, increased glycemic variability, male sex, underlying comorbidities, and pre-existing complications such as sub-clinical atherosclerosis or albuminuria are all at a heightened risk of microvascular and macrovascular complications, which can have a significant impact on life quality^{217–219}.

Mechanisms Leading to Diabetes Complications

A primary mechanism through which hyperglycemia induces cellular damage is via increased **oxidative stress**. Elevated glucose levels foster ROS overproduction in various cell types besides β -cells, including endothelial cells, vascular smooth muscle cells, and neurons^{220,221}. Excessive ROS production can overwhelm cellular antioxidant defenses, culminating in oxidative damage to proteins, lipids, and nucleic acids, ultimately impairing cellular function in these cell types²²². Furthermore, hyperglycemia-induced mitochondrial dysfunction has been implicated in increased oxidative stress, as dysfunctional mitochondria constitute a significant source of ROS²²³.

Another essential factor exacerbating diabetes complications is the accumulation of **advanced glycation end products (AGEs)**. These encompass a broad spectrum of compounds generated from non-enzymatic glycation reactions between reducing carbohydrates and free amino groups in proteins, lipids, and nucleic acids²²⁴. Chronic hyperglycemia accelerates the formation and accumulation of AGEs, which can provoke structural and functional alterations in various tissues. For instance, AGEs can cross-link with extracellular matrix proteins, such as collagen and elastin, impairing their function and contributing to the development of microvascular and macrovascular complications²²⁵. AGEs can also interact with specific cell surface receptors, such as the receptor for AGEs, leading to the activation of inflammatory and pro-oxidant pathways²²⁶.

Inflammation is another critical player in hyperglycemia-induced tissue damage. Elevated glucose levels can activate several pro-inflammatory signaling pathways, such as NF- κ B and MAPK pathways, resulting in the increased production of pro-inflammatory cytokines and chemokines²²⁷. Inflammatory mediators, in turn, can exacerbate oxidative stress, promote endothelial dysfunction, and contribute to the development and progression of diabetes complications²²⁸.

Overall, hyperglycemia-induced cellular and tissue damage in diabetes is a multifaceted process involving oxidative stress, the formation and accumulation of AGEs, and the activation of inflammatory pathways.

Clinical Manifestations of Diabetes Complications

Microvascular complications primarily stem from damage to the small blood vessels, encompassing diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy. Diabetic retinopathy, a leading cause of preventable blindness worldwide, is characterized by vascular abnormalities within the retinal microvasculature, leading to progressive vision loss²²⁹. Hyperglycemia-induced oxidative stress, inflammation, and endothelial dysfunction are among the key factors driving the development of diabetic retinopathy²³⁰. Diabetic nephropathy, a major contributor to end-stage renal disease, is characterized by a gradual decline in renal function due to glomerular and tubulointerstitial damage²³¹. Hyperglycemia, hypertension, and genetic predisposition are significant risk factors for diabetic nephropathy, with the renin-angiotensin-aldosterone system playing a central role in its progression²³². Diabetic neuropathy, a heterogeneous group of disorders affecting peripheral nerves, manifests as diverse symptoms ranging from pain and paresthesias to autonomic dysfunction²³³. The pathogenesis of diabetic neuropathy is multifactorial, involving

metabolic, vascular, and immune-mediated mechanisms, with hyperglycemia-induced oxidative stress and mitochondrial dysfunction as key contributors²³⁴.

Macrovascular complications of diabetes include coronary artery disease, peripheral artery disease, and cerebrovascular disease, which can lead to myocardial infarction, limb ischemia, and stroke, respectively²³⁵. These complications are primarily driven by atherosclerosis, a chronic inflammatory process characterized by endothelial dysfunction, lipid deposition, and plaque formation within the arterial wall²³⁶. Diabetes accelerates atherosclerosis through multiple mechanisms, including dyslipidemia, oxidative stress, inflammation, and AGEs accumulation²²².

Beyond the issues mentioned above, diabetes can lead to **further complications** affecting the skin, gastrointestinal tract, and musculoskeletal system. For instance, diabetic dermopathy, characterized by skin lesions and poor wound healing, can result from a combination of factors such as microangiopathy, immune dysfunction, and neuropathy²³⁷. Gastrointestinal complications, including gastroparesis, enteropathy, and constipation, can be attributed to diabetic autonomic neuropathy and alterations in gut motility²³⁸. Moreover, musculoskeletal complications, such as joint inflammation, amyotrophy, and osteoporosis, can arise from the increase in AGEs²³⁹.

Diabetes complications can also adversely impact **mental health**, as individuals with diabetes have a higher prevalence of depression, anxiety, and cognitive decline¹³⁸. The complex interplay between biological, psychological, and social factors is thought to contribute to the increased risk of mental health disorders in this population²⁴⁰.

Achieving intensive glycemic control, particularly through early intervention, could have a clinically significant impact in reducing the risk of future complications^{241,242}. However, for patients with long-term T2D, the intensification of glucose-lowering therapy at a later stage may not be advantageous and could, in fact, result in worse outcomes^{243,244}. Preventing and managing diabetes complications necessitate a comprehensive, multidisciplinary approach, incorporating optimal glycemic control, risk factor modification, and individualized treatment strategies. Furthermore, addressing mental health and quality of life in individuals with diabetes is crucial for achieving better long-term outcomes²⁴⁵.

1.2.3. Prevention and Treatment of Type 2 Diabetes

Screening the general population for diabetes is not recommended due to insufficient evidence of its cost-effectiveness or health benefits. Instead, experts recommend targeted screening of high-risk individuals, such as those who are overweight or obese, belong to ethnic groups with a high susceptibility to T2D at an early age or have a family history of T2D and a history of gestational diabetes^{246,247}. When a diagnosis of T2D is suspected, plasma glucose concentrations or HbA1c levels can be analyzed, and OGTTs can also be performed to confirm the diagnosis. Differentiating T2D from other types of diabetes is not always straightforward and requires a careful review of the patient's history and clinical context¹³⁸.

Early proactive management is crucial in delaying or preventing the onset of T2D in high-risk individuals. Lifestyle interventions, such as calorie-restricted diets and exercise, that target weight loss and the use of pharmacotherapy have been shown to prevent or delay the onset of T2D^{138,248}. Moreover, community-based cluster phenotyping could assist in identifying people at a higher risk for T2D and enable efficient prevention strategies²⁴⁹. However, long-term adherence and effectiveness of these interventions can be challenging, particularly in low-income and middle-income countries^{250,251}. Risk assessment and management of high-risk subjects (those that present fasting plasma glucose of 5.5-6.9 mM or HbA1c of 6.0-6.4%) are the key components of this strategy. Overweight individuals with a BMI of 25 kg·m⁻² or more (≥ 23 kg·m⁻² in some ethnic groups) are encouraged to lose weight through diet, exercise, and pharmacotherapies¹³⁸. Modern technology, including wearable devices, could help deliver prevention programs^{252,253}. A systematic review assessed the effectiveness of various technology-driven programs to prevent T2D, with digital features including diet and weight tracking, activity monitors, and reminders. These technology-driven programs were found to be effective in both the short- and long-term²⁵⁴.

The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) guidelines recommend a target **HbA1c of 7% or less** for most adults with T2D, as it can provide long-term benefits, especially in preventing microvascular complications. A comprehensive approach focusing on weight reduction, cardiovascular risk factor control, and cardiorenal protection is encouraged instead of solely targeting blood glucose control. Moreover, managing comorbidities is vital for reducing complications and improving the quality of life for T2D

patients²⁵⁵. Equally important, remission should be a treatment goal for those newly diagnosed or with a short disease duration¹³⁸.

Lifestyle interventions, including healthy eating, regular exercise, weight management, and smoking cessation, are crucial for long-term T2D management. These lifestyle modifications can enhance glycemic control, decrease cardiovascular disease risk, and improve the overall quality of life¹³⁸. Given that over 80% of T2D patients are overweight or obese, **weight loss** becomes a significant strategy in reducing the metabolic burden of diabetes²⁵⁶. In line with this, it has been observed that a 5-10% weight loss can delay the progression from prediabetes to diabetes, while a 15% or more weight loss can induce diabetes remission²⁴⁸. Achieving weight loss through a **balanced and low-energy diet** is key to short-term T2D remission, but long-term adherence is challenging²⁵⁷. Emerging T2D management strategies include ketogenic diets and intermittent fasting. Ketogenic diets primarily consist of consuming 90% of daily calories from fat, with only 10% coming from proteins and carbohydrates. Intermittent fasting, on the other hand, is a dietary pattern characterized by alternating periods of very low or no calorie intake with periods of unrestricted eating. However, while these dietary approaches show promise, their full potential in managing T2D is yet to be determined²⁵⁸⁻²⁶⁰. **Exercise** is another vital component of T2D management, offering numerous metabolic benefits beyond glycemic control through energy expenditure, such as improved insulin sensitivity, reduced visceral fat content, enhanced mental well-being, and significantly lower cardiovascular and overall mortality risk²⁶¹⁻²⁶³.

While non-pharmacological therapies are typically the initial approach for managing T2D, **medication** also constitutes an integral component of the overall treatment regimen¹³⁸. **Metformin** is generally considered the first-line pharmacotherapy for T2D patients due to its low cost, availability, ease of administration, good tolerability, low hypoglycemia risk, weight neutrality, combinability, and long-standing clinical efficacy⁹⁸. Metformin acts by activating adenosine monophosphate-activated protein kinase (AMPK), a key enzyme that regulates energy metabolism in cells²⁶⁴. Through this pathway, metformin decreases hepatic gluconeogenesis and increases glucose uptake and utilization in peripheral tissues, leading to a reduction in blood glucose levels²⁶⁵. Additionally, metformin may improve insulin sensitivity by enhancing insulin signaling pathways in cells²⁶⁴.

For individuals with established or high-risk cardiovascular disease, heart failure, or chronic kidney disease, it is recommended to initiate treatment with sodium-glucose cotransporter 2 (**SGLT2**) **inhibitors**, with or without other medication^{98,266}. SGLT2 inhibitors reduce glucose reabsorption in the kidneys, promoting glucose excretion through urine²⁶⁷.

Sulfonylureas and meglitinides are affordable oral medications used for treating T2D¹³⁸. **Sulfonylureas** achieve K_{ATP} channel closure in β -cells by binding to and blocking the sulfonylurea receptor (SUR), a K_{ATP} channel complex regulatory subunit. This blocks the outward flow of potassium ions, leading to cell membrane depolarization and the subsequent release of insulin-containing granules^{268,269}. **Meglitinides** function similarly but with a weaker binding to SUR, resulting in a shorter duration of action²⁶⁹.

Thiazolidinediones, such as pioglitazone, are peroxisome proliferator-activated receptor gamma agonists that provide long-lasting glucose-lowering properties by enhancing insulin sensitivity in peripheral tissues and inhibiting hepatic glucose production^{138,270}. However, their side effects, including fluid retention, weight gain, heart failure, and decreased bone mineral density, warrant consideration¹³⁸.

Targeting incretin hormones has become a pivotal strategy in treating T2D, mainly through the **modulation of GLP-1R activity**^{94,138}. GLP-1R agonists, such as exenatide and liraglutide, exhibit increased resistance to enzymatic degradation by DPP-4. The utilization of these GLP-1 analogs brings multiple advantages, including the potential promotion of weight loss and amelioration of metabolic dysfunction^{98,138,269}. Although DPP-4 inhibitors have gained popularity due to their safety profile, they only offer modest glucose-lowering efficacy and do not significantly impact weight¹³⁸.

Notwithstanding, **insulin therapy** remains a central component of T2D management, particularly for patients unable to reach glycemic targets with other therapies. Insulin therapy can include basal insulin, which provides a steady level of insulin throughout the day, or bolus insulin, which delivers a rapid-acting insulin dose before meals to help control postprandial glucose levels^{98,138}.

Metabolic surgery is a highly effective yet invasive procedure for achieving sustained weight loss and T2D remission in certain cases^{138,271}. Nevertheless, it is associated with complications such as nutritional deficiencies and postprandial hypoglycemia. Consequently, it should be considered an essential addition to pharmaco-

therapy and lifestyle intervention options but only for select patients with morbid obesity¹³⁸.

To sum up, addressing the complexities of T2D management requires a holistic and multifaceted approach, encompassing targeted screening, timely intervention, lifestyle modifications, and judicious selection of pharmacological therapies. The integration of technology-driven programs and the adoption of innovative management strategies hold the potential to bolster the efficacy of T2D prevention and treatment measures. A comprehensive, patient-centric approach that emphasizes weight reduction, cardiovascular risk factor management, cardiorenal protection, and the handling of comorbidities is paramount in mitigating complications and enhancing the quality of life for individuals with T2D. However, it is crucial to note that current pharmacological and surgical interventions are not exempt from secondary effects, which can be concerning in some instances. Despite advances in the management of T2D, there is still no definitive cure for the disease^{98,138}. The alarming increase in the global incidence and prevalence of T2D, particularly in the face of the growing burden on healthcare systems and the associated socioeconomic costs, make finding new treatments for T2D compelling.

1.3. The Succinate-Succinate Receptor 1 Axis

Succinate, a key intermediate metabolite in the TCA cycle (**Figure 8**), has garnered considerable attention in recent years due to its multifaceted roles in cellular metabolism, signaling pathways, and regulation of diverse physiological and pathological processes²⁷². Structurally, succinate is the anionic form of succinic acid, a dicarboxylic acid with the chemical formula $C_4H_6O_4$. Its critical involvement in energy production and cellular respiration has made it a central focus of research in biochemistry and metabolism²⁷³.

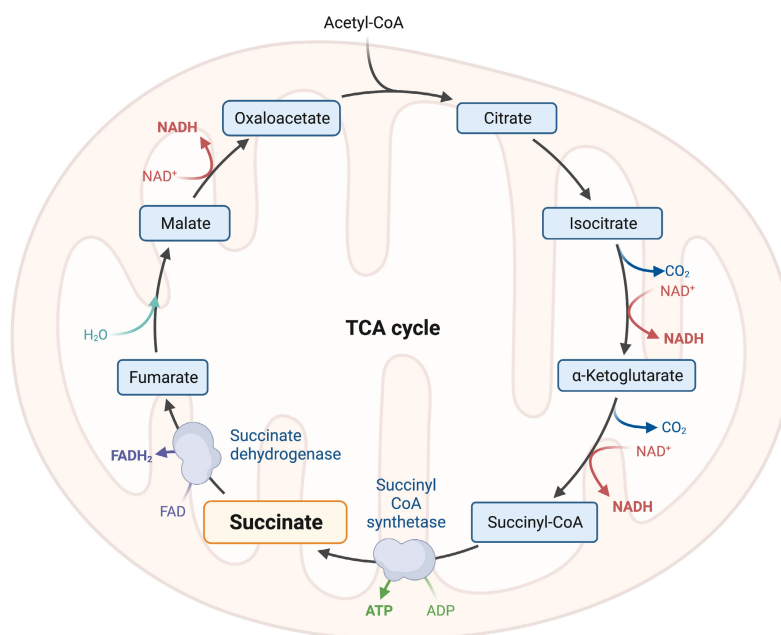


Figure 8. Schematic Representation of the Tricarboxylic Acid (TCA) Cycle. This diagram delineates the key metabolites and their interconversions within the TCA cycle in the mitochondria. Specific emphasis is placed on succinate synthesis and its subsequent conversion into fumarate, detailing the catalyzing enzymes involved in these steps. ADP: adenosine diphosphate; ATP: adenosine triphosphate; CoA: coenzyme A; FAD: flavin adenine dinucleotide; NAD: nicotinamide adenine dinucleotide.

Historically, the discovery of succinate's function in **energy metabolism** can be traced back to the early 20th century when Hans Adolf Krebs, a German-British biochemist, elucidated the TCA cycle, also known as the Krebs cycle or the citric acid cycle²⁷³. This cycle represents a series of chemical reactions utilized by all aerobic organisms to release stored energy through acetyl-CoA oxidation derived from carbohydrates, lipids, and proteins²⁷⁴. The TCA cycle consists of eight distinct enzymatic reactions that occur within the mitochondrial matrix. In the fourth step of

the cycle, α -ketoglutarate (α -KG) is converted to succinyl-CoA through an oxidative decarboxylation reaction catalyzed by the α -KG dehydrogenase complex. This reaction generates one molecule of NADH and CO_2 . Subsequently, succinyl-CoA is converted to succinate in the fifth step of the TCA cycle, catalyzed by succinyl-CoA synthetase²⁷⁵. This reaction results in the formation of one molecule of guanosine triphosphate (GTP) or ATP, depending on the specific isoform of the enzyme expressed in the organism²⁷⁶. Notably, this is the only substrate-level phosphorylation event in the TCA cycle, providing a direct energy source. The conversion of succinate to fumarate, the sixth step in the TCA cycle, is catalyzed by succinate dehydrogenase (SDH), which is embedded within the inner mitochondrial membrane. This enzyme also constitutes the complex II of the electron transport chain (ETC). The oxidation of succinate to fumarate generates one molecule of FADH_2 , which is subsequently utilized in the ETC to contribute to the proton gradient driving ATP synthesis through oxidative phosphorylation²⁷⁵.

While the indispensable role of succinate in the TCA cycle and energy metabolism has been well-established, recent research has unveiled a myriad of additional functions that underscore its significance in **cellular signaling** and regulation²⁷². Notably, the discovery of succinate as the endogenous ligand for a formerly orphan G protein-coupled receptor, now classified as **succinate receptor 1 (SUCNR1)**, shed light on an extensive range of extracellular signaling capabilities and potential influences on various pathophysiological processes²⁷⁷. Despite these breakthroughs, a complete understanding of the role of the succinate-SUCNR1 axis in cellular processes has yet to be achieved, hinting at the possibility of even more undiscovered actions. Succinate's intracellular roles, export, and eventual engagement with SUCNR1 are visually summarized in **Figure 9**, providing a schematic overview of this axis.

1.3.1. Intracellular Roles of Succinate Beyond Energy Metabolism

In addition to its role in energy production, succinate serves as a versatile signaling molecule with both intracellular and extracellular functions. Intracellularly, **succinate accumulation** has been associated with aerobic glycolysis, TCA cycle disruption, and anaplerosis, processes that can be connected to hypoxia, immune cell activation, and tumorigenesis^{278–280}. However, the range of succinate's functions extends beyond these pathological conditions to include normal physiological responses²⁷². These connections have significant implications for various biological processes,

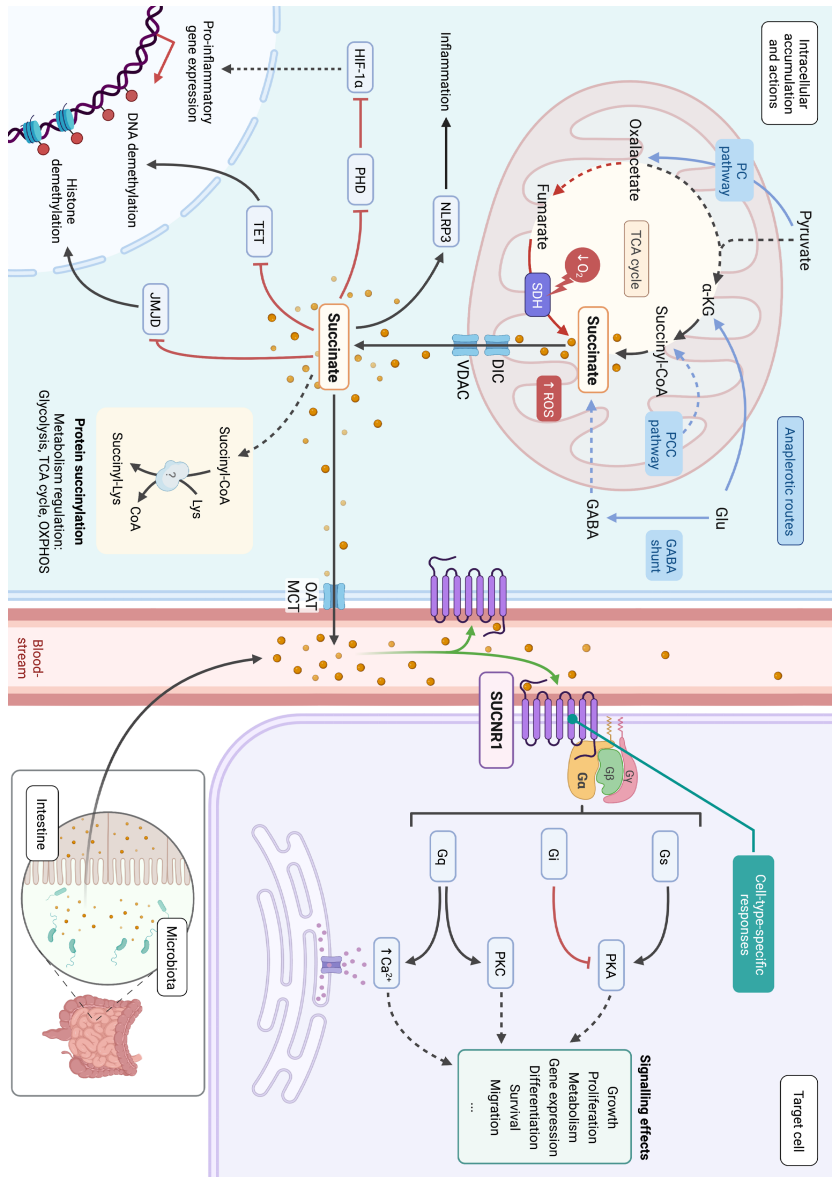


Figure 9. Overview of the Succinate-SUCNR1 Axis. This schematic initiates with succinate accumulation within the mitochondria, attributed to the combined effects of the tricarboxylic acid (TCA) cycle, anaplerotic routes, and the reverse action of succinate dehydrogenase (SDH) in hypoxia. The ensuing accumulation stimulates the production of reactive oxygen species (ROS). Then, succinate is transported to the cytosol, where it assumes various signaling roles. Additionally, an important source of succinate is the gut, which significantly contributes to systemic levels. Upon reaching the extracellular space, succinate activates the succinate receptor 1 (SUCNR1), inciting a spectrum of cell-type-specific downstream responses. The reported Gα proteins known to interact with SUCNR1 are shown: α-KG: α-ketoglutarate; DIC: dicarboxylate carrier; GABA: γ-aminobutyric acid; Glu: glutamate; HIF-1α: hypoxia-inducible factor 1 alpha; JMJD: jumoni C-domain-containing protein; Lys: lysine; MCT: monocarboxylate transporter; NLRP3: NACHT, LRR and PYD domains-containing protein 3 inflammasome; OAT: organic anion/dicarboxylate transporter; OXPHOS: oxidative phosphorylation; PC: pyruvate carboxylase; PCC: propionyl-CoA carboxylase; PHD: prolyl/hydroxylase domain enzyme; PKA: protein kinase A; PKC: protein kinase C; TET: ten-eleven translocation protein; VDAC: voltage-dependent anion channel.

highlighting the importance of understanding the intricate interplay between succinate and cellular processes.

1.3.1.1. Mechanisms of Intracellular Succinate Accumulation

Mitochondrial metabolism serves as the primary source of succinate generation under physiological conditions, with the TCA cycle playing a central role in this process. However, under cellular stress or hypoxia, alternative metabolic pathways and anaplerotic routes may come into play, contributing to succinate accumulation^{279,280}. One such pathway involves the reverse activity of SDH, which has been implicated in the accumulation of succinate under stress conditions, primarily hypoxia. Under these conditions, the ETC becomes impaired, leading to a buildup of reducing equivalents in the form of NADH and FADH₂. This increase in reducing equivalents drives the reverse electron transfer from complex II to complex I, ultimately leading to the reduction of fumarate to succinate²⁸¹. This process significantly contributes to succinate accumulation under hypoxia or stress conditions and is linked to cellular responses such as hypoxia-inducible factor-1 α (HIF-1 α) stabilization and the production of ROS^{280,281}.

Anaplerotic routes, which replenish TCA cycle intermediates, can also contribute to intracellular succinate accumulation^{272,282}. For instance, PC catalyzes the conversion of pyruvate to oxaloacetate, an important anaplerotic reaction that can indirectly increase succinate levels through its impact on the TCA cycle²⁸³. Additionally, the propionyl-CoA carboxylase pathway, involved in the catabolism of branched-chain amino acids and odd-chain fatty acids, generates succinyl-CoA, which can be converted to succinate²⁸⁴. Moreover, increased glutamine metabolism can result in succinate accumulation through anaplerosis of α -KG into the TCA cycle²⁷⁹. Another important mechanism contributing to intracellular succinate accumulation is the upregulation of the γ -aminobutyric acid (GABA) shunt, which links the metabolism of glutamate and GABA^{278,279}. This metabolic pathway converts glutamate into GABA through the action of glutamate decarboxylase and subsequently transforms GABA into succinate *via* intermediate steps, thus contributing to its accumulation²⁸⁵.

Intriguingly, recent studies have demonstrated that certain PTMs can influence TCA cycle enzyme activities and subsequently modulate succinate levels. One such example is SDH acetylation, which reduces the enzyme's activity, thereby increasing succinate accumulation²⁸⁶. Notably, the increase in intracellular succinate due to aberrant SDH activity has been observed in pathological contexts. For example, mu-

tations in SDH and other TCA cycle enzymes have been linked to hereditary tumor syndromes and various forms of cancer²⁸⁷.

Overall, the mechanisms driving intracellular succinate accumulation are multifarious and context-dependent. These mechanisms encompass canonical metabolic pathways, anaplerotic routes, alternative routes under stress conditions, and enzyme regulation.

1.3.1.2. Intracellular Signaling Properties of Succinate

Elevated intracellular succinate levels drive diverse effects in cellular signaling, such as protein succinylation²⁸⁸, stabilization of HIF-1 α ^{278,280}, and increased production of mitochondrial ROS^{279,289}. These effects prompt cellular stress responses and activate metabolic reprogramming pathways. Notably, the elevated production of mitochondrial ROS is a vital driver of these processes and is directly linked to intracellular succinate accumulation^{279,280,289}.

One major mechanism connecting intracellular succinate accumulation to inflammation is the **stabilization and activation of HIF-1 α** , a transcription factor that regulates the expression of genes involved in various cellular processes, such as angiogenesis and inflammation^{279,290,291}. Under normoxic conditions, HIF-1 α is hydroxylated by prolyl hydroxylase domain (PHD) enzymes, facilitating its degradation²⁹². However, accumulating intracellular succinate has been shown to inhibit PHD enzymes, leading to HIF-1 α stabilization and promoting its transcriptional activity²⁹³. As a result, HIF-1 α activation induces the expression of pro-inflammatory genes, such as those encoding IL-1 β and inducible nitric oxide synthase^{278,294}.

Furthermore, another molecular mechanism linking intracellular succinate to inflammation involves the activation of the NACHT, LRR, and PYD domains-containing protein 3 (**NLRP3**) **inflammasome**, which is a multiprotein complex responsible for the activation of caspase-1, which subsequently leads to the cleavage and maturation of pro-inflammatory cytokines, including IL-1 β and interleukin 18²⁹⁵. Succinate has been demonstrated to promote the assembly and activation of the NLRP3 inflammasome by enhancing the production of ROS^{291,295,296}.

Succinate can also influence gene expression, with a significant aspect being the interplay between intracellular succinate and **epigenetic regulation** by the inhibition of α -KG-dependent dioxygenases, such as Jumonji C-domain-containing histone demethylases and the ten-eleven translocation family of DNA demethylas-

es²⁹⁷⁻³⁰⁰. These enzymes require α -KG as a co-substrate and are competitively inhibited by accumulating succinate, leading to global changes in histone and DNA methylation^{298,300}. Consequently, this results in the stabilization of various histone and DNA methylation marks induced by metabolic reprogramming, which can modulate gene expression and therefore impact cellular function^{301,302}.

Protein succinylation, a PTM involving the addition of a succinyl group to lysine residues within proteins, has recently garnered considerable attention due to its potential regulatory role in cellular metabolism and signaling pathways. Recent advancements in proteomic techniques, especially mass spectrometry, have expanded the identification of succinylated proteins and their functional roles^{288,303}. Succinylation affects various cellular processes by altering protein location, stability, folding, and enzymatic activity^{303,304}. Remarkably, this PTM plays a crucial role in regulating cellular metabolism, including glycolysis, the TCA cycle, and oxidative phosphorylation^{305,306}. Dysregulation of protein succinylation has been associated with detrimental effects on cellular metabolism, as it can alter enzyme kinetics and substrate binding, thus disrupting the overall metabolic flux within cells^{303,307,308}.

The effects of succinylation on enzymatic activity are specific to the target protein and lysine residue modified. Intriguingly, SDH can be succinylated, leading to an increase in enzymatic activity³⁰⁶. Additionally, the interplay between succinylation and other PTMs, such as acetylation, phosphorylation, and ubiquitination, is essential for fine-tuning metabolic pathways and epigenetic regulation^{302,303,309}.

While the mechanism by which succinylation occurs remains elusive^{310,311}, sirtuin 5 (SIRT5) has emerged as a significant enzyme responsible for removing succinyl groups from proteins^{306,308}. SIRT5's desuccinylase activity is regulated by the NAD⁺/NADH ratio, linking the cellular metabolic state to protein succinylation dynamics³¹². Dysregulation of protein succinylation and its associated enzymes, such as SIRT5, have been implicated in various diseases, including cancer, cardiovascular diseases, and neurological disorders³¹⁰.

1.3.1.3. Role of Intracellular Succinate in Regulating Insulin Secretion

Although substantial research on intracellular succinate's role in modulating insulin secretion in β -cells was conducted previously, interest in this subject has seemingly waned in recent years. Nonetheless, earlier findings continue to offer valuable insights into the mechanisms underlying intracellular succinate's influence on insulin release, meriting a thorough examination and discussion.

A critical observation from previous research indicates that, in the absence of glucose exposure, succinate does not stimulate insulin release³¹³. As a result, subsequent investigations have centered on using **cell-permeable succinate esters** to elevate intracellular succinate levels in β -cells. These esters, such as dimethyl succinate and diethyl succinate, can diffuse through the plasma membrane without needing specific transporters, in contrast to succinate itself³¹⁴. This distinct characteristic allows succinate esters to rapidly enter β -cells and increase intracellular succinate concentrations when processed by esterases. Until now, it has been proposed that succinate's insulinotropic effects are reliant on its uptake, highlighting the importance of permeable succinate esters and emphasizing the significance of intracellular succinate in insulin secretion regulation when acting primarily as a metabolic fuel and driving insulin release through ATP generation^{66,315,316}.

Exogenous esters of succinate, at 10 mM, are potent insulin secretagogues, outperforming other compounds like malate, α -KG, fumarate esters, and citrate esters in this function^{315,317,318}. Compared to other TCA cycle metabolites, the intracellular succinate's unique insulinotropic properties stem from various reasons. Firstly, its ability to interact with SDH, which directly links to ATP generation; secondly, succinate synthetase activity (which can function reversely to produce succinyl-CoA) combined with succinyl-CoA-acetoacetate transferase, which generate acetoacetyl (AcAc)-CoA from succinate. AcAc-CoA, in turn, can be converted into mevalonate through hydroxy-3 methylglutaryl CoA (HMG-CoA) synthetase and HMG-CoA reductase reactions, with mevalonate or its derivatives potentially serving as insulin release signals³¹⁸⁻³¹⁹.

Moreover, in rat islet mitochondria, SDH oxidizes succinate to fumarate, which is subsequently transformed into malate by fumarase. Malate then enters the cytosol, where the malic enzyme converts it to pyruvate. Subsequently, pyruvate can re-enter the mitochondria after PDH and PC reactions, which convert pyruvate into acetyl-CoA and oxaloacetate, respectively³¹⁸. Additionally, succinate metabolism bolsters insulin release *via* increased NADPH production by the malic enzyme,

which in turn elevates mevalonate production through HMG-CoA reductase. The importance of the malic enzyme is highlighted by the lack of response to succinate as a secretagogue in mouse islets naturally devoid of malic enzyme activity, unlike rat islets^{318,320}.

Remarkably, succinate has also been identified as a preferential metabolic stimulus-coupling signal for glucose-induced insulin biosynthesis independently of insulin secretion. Accordingly, succinate can specifically stimulate preproinsulin transcription and translation without affecting insulin secretion. As a result, disruptions in intracellular succinate-mediated signaling pathways could potentially lead to alterations in insulin content, ultimately impacting insulin secretion and glucose homeostasis^{321,322}.

A thorough analysis of metabolites associated with glucose-stimulated biphasic insulin secretion further emphasized the importance of succinate insulin secretion regulation. In line with this, intracellular succinate levels increase in rat β -cells in response to glucose, and these levels were found to be the most positively associated with insulin secretion along with α -KG and pyruvate³²³.

Overall, intracellular succinate plays a crucial role in modulating insulin secretion in β -cells. Using cell-permeable succinate esters has been instrumental in understanding succinate's insulintropic effects. The existing literature points to several mechanisms, including mitochondrial activation, modulation of metabolic pathways, and insulin biosynthesis, through which intracellular succinate influences insulin secretion. Despite the valuable insights gained from earlier research, there is a noticeable lack of studies exploring the role of extracellular succinate under glucose conditions and its potential involvement with SUCNR1 in β -cells, which remains unexplored.

1.3.2. Succinate's Transport Across Membranes

The regulation of extracellular succinate concentrations is of notable significance, particularly considering its function as a signaling molecule. Succinate's traversal across the plasma membrane presents a unique quandary stemming from its inherent chemical properties. Due to its anionic nature and hydrophilic characteristics, succinate cannot passively diffuse through lipid bilayers³¹⁴, necessitating the involvement of specialized transport systems to facilitate its translocation²⁷².

Emerging evidence suggests that specific **transporters** may facilitate the movement of succinate across cell membranes. While the precise mechanism governing succinate release and uptake is yet to be fully elucidated, current research implies that it is under stringent control²⁷². Although essential for succinate transport, these carriers demonstrate substrate flexibility, enabling the transport of other molecules as well. In the mitochondria, the **dicarboxylate carrier (DIC)**, which belongs to the solute carrier (SLC) family 25 (SLC25) and is located in the inner membrane, and the **voltage-dependent anion channel (VDAC)**, which is situated in the outer membrane, mediate succinate efflux from the mitochondrial matrix^{272,324}, contributing to its cytosol accumulation. Succinate release into the extracellular environment can occur through organic anion/dicarboxylate transporters (OATs)³²⁵ and monocarboxylate transporters (MCTs)³²⁶, while sodium-dependent dicarboxylate transporters (NaDC) can mediate its uptake^{327,328}.

OATs, which are members of the SLC22 family, play a vital role in transporting small metabolites, drugs, and toxins, particularly in the liver and kidney³²⁹. They function as exchangers for the inward transport of organic anions in exchange for metabolic product extrusion into circulation. Multiple subtypes of OATs have been identified and characterized, with varying substrate preferences, tissue location, and regulation. Specifically, OAT1-OAT3, OAT5, and OAT10 can exchange organic anions with succinate as a counteranion^{330,331}.

MCTs belong to the SLC16 family and are pivotal for transporting short-chain monocarboxylates, nutrients, hormones, and amino acids in a proton-linked manner. Succinate can serve as a substrate for MCT1 in its protonated monocarboxylate form³²⁶.

The **NaDC family** can facilitate succinate uptake. Specifically, NaDC1 is predominantly expressed in the kidney and intestine³²⁸. In the kidney, it reabsorbs TCA intermediates from the tubular filtrate, while in the intestine it uptakes digested nutrients. The kidney's reabsorption of succinate seems to be associated with blood pressure control³³². Moreover, NaDC3 (encoded by *SLC13A3*) has a broader tissue distribution and higher substrate affinity than NaDC1. Single-nucleotide polymorphisms of *SLC13A3* are linked to the development of T2D through an unknown mechanism³³³. Interestingly, succinate uptake by NaDC1 and NaDC3 is inhibited by SUCNR1 activation in renal cells³³², indicating an interplay between intracellular/extracellular succinate levels regulation and succinate sensing by SUCNR1 to regulate these levels. Succinate can also be transported by the Na⁺/citrate cotransporter (NaCT), which is predominantly located in the liver and brain, although with lower affinity^{328,334}.

Notwithstanding, the precise mechanisms governing succinate export and their regulation remain largely enigmatic, warranting further investigation to elucidate the factors influencing extracellular and circulating succinate levels.

1.3.3. Sources and Implications of Circulating Succinate in Health and Disease

In normal physiological conditions, initial studies reported 2-3 μM circulating succinate levels using liquid chromatography-tandem mass spectrometry³³⁵. However, more recent observations using similar technology^{336,337} detected concentrations comparable to those obtained by enzymatic assay kits, which have established that plasma and serum concentrations of succinate might be in the range of 5-200 μM ³³⁸⁻³⁴⁰.

In addition to intracellular sources, circulating succinate levels are influenced by extracellular factors. For instance, the **gut microbiome** is implicated in succinate production, as various microbial species produce succinate as a fermentation by-product. Until recently, succinate was primarily considered an intermediary compound for energy production within the gut microbiota, and its functional implications received limited attention³⁴¹. Nevertheless, recent research has shown that dietary intake and absorption of succinate from food sources also contribute to circulating succinate levels³⁴². In humans, succinate accounts for 2-4% of total organic anions, ranging from 1 to 3 mM, in intestinal content and feces³⁴³. Succinate serves as both a precursor of propionate and a common end-product of some bacteria. It is a primary cross-feeding metabolite between gut resident microbes, produced by some primary fermenters and consumed by secondary fermenters. While mainly formed from carbohydrate fermentation, succinate can also arise from amino acid fermentation. There is no standard classification of succinate producers and consumers due to the complexity of cross-feeding relationships *in vitro*.

Nonetheless, some bacterial species have been identified to excrete succinate (e.g., *Prevotella copri*, *Bacteroides fragilis*, and *Succinivibrio dextrinosolvens*), while others consume it (e.g., *Bacteroides thetaiotaomicron*, *Phascolarctobacterium succinatutens*, and *Dialister succinatiphilus*)³⁴². Unlike short-chain fatty acids, succinate is present in low concentrations in the gut lumen, likely due to its cross-feeding relationships. However, in conditions such as inflammatory bowel disease and obesity, succinate accumulation in the intestinal lumen increases, indicating a potential link to gut dysbiosis^{338,344}. Probiotic interventions with bacterial succinate producers have proved

effective for improving metabolic abnormalities associated with obesity and insulin resistance^{345–347}. While succinate is gaining interest as a microbial-derived metabolite due to its role in gut homeostasis, pathogen susceptibility, and inflammation-related diseases such as obesity³⁴², it was only recently that we established the gut microbiota as a substantial contributor to circulating succinate levels, especially in the obesity context³⁴⁷.

Overall, the sources of circulating succinate are diverse and context-dependent, encompassing mitochondrial metabolism, alternative pathways under cellular stress, gut microbiota, and dietary intake. Further exploration of the intricate relationship between succinate and various pathophysiological states promises to unlock new targeted therapeutic interventions and advance our comprehension of metabolic regulation.

1.3.3.1. Circulating Succinate in Health

In recent years, the study of succinate's role in physiological conditions such as physical exercise, food ingestion, and cold exposure has broadened our understanding of its multifaceted contributions to human health²⁷².

Physical exercise represents a physiological condition during which the body undergoes several metabolic adaptations to meet the increased energy demands of working muscles. Previously, it was reported that circulating succinate levels increase during anaerobic endurance exercise³⁴⁸. Moreover, it was demonstrated that succinate could improve oxygen consumption in skeletal muscle under pathological conditions associated with mitochondrial dysfunction³⁴⁹. Exercise induces a unique metabolite signature in skeletal muscle, including an increase in succinate, which has been linked to improvements in insulin sensitivity³⁵⁰. Interestingly, the increase in muscle activity is associated with an accumulation of large amounts of succinate in arterial blood, suggesting that succinate might regulate the hepatic exercise response. This adaptation involves an increase in hepatic glucose production and fat oxidation, which are crucial for meeting the energy demands of exercise. Given these findings, it has been suggested that succinate could be used as a dietary supplement to improve physiological fitness and counteract fatigue³⁵¹. Recently, the role of circulating succinate during exercise was further explored, demonstrating that extracellular succinate acts as a metabolic signal to promote angiogenesis and innervation in skeletal muscles, thereby enhancing blood flow and oxygen supply and participating in muscle remodeling after exercise. The researchers found that

pH-gated succinate secretion regulates muscle remodeling in response to exercise, highlighting the importance of circulating succinate in exercise physiology and adaptation³²⁶.

Food ingestion and the host organism's subsequent metabolic processing of nutrients can also modulate circulating succinate levels. In line with this, we have recently presented a novel perspective on plasma succinate, demonstrating its **nutritional modulation** by luminal nutrients and its connection to an individual's metabolic status. In patients with morbid obesity and T2D, this meal-related response of circulating succinate is blunted. However, after weight loss following bariatric surgery, the response recovers, pointing to a potential role for succinate in metabolic homeostasis. Additionally, glucose transit through the intestinal tract affects postprandial succinate dynamics, indicating that the intestine may be a significant source of circulating succinate after feeding. Although the precise physiological role of nutrient-related succinate dynamics remains to be determined, we suggested that succinate could function similarly to other hormones, acting as a paracrine and autocrine signal in metabolic tissues. Our data also unveiled a role for succinate in healthy metabolic homeostasis, contrary to its previous association exclusively with hypoxia, tissue damage, and inflammation^{272,352}.

Cold exposure is another physiological condition recently associated with alterations in bloodstream succinate. During cold exposure, non-shivering thermogenesis is initiated in brown adipose tissue (BAT) to maintain body temperature³⁵³. Intriguingly, it was found that cold exposure increases circulating succinate levels in mice. In BAT, the accumulation of succinate is driven by the capacity of brown adipocytes to scavenge extracellular succinate, rapidly uptaking and employing it to increase uncoupling protein 1 (UCP1)-dependent thermogenic respiration through SDH mediated-succinate oxidation and subsequent ROS production³³⁶. Moreover, succinate has been identified as an inductor of beige adipogenesis in white adipocyte progenitors³⁵⁴. In accordance with this function, dietary supplementation with succinate increases energy expenditure and may protect against diet-induced obesity^{336,354}.

1.3.2.2. Circulating Succinate in Metabolic Diseases

Circulating succinate levels are increased in numerous chronic inflammatory diseases, including metabolic pathologies²⁷². Diverging from the transient elevation of extracellular succinate concentrations observed during physiological adaptations, pathological states such as obesity, T2D, cardiovascular disease, and hepatic disorders are associated with sustained elevations of extracellular succinate levels^{338,339,355,356}. The clinical relevance of these persistent increases has been established through various studies in the context of these diseases.

Since **obesity** and **T2D** are closely related, it is sometimes difficult to differentiate the metabolic disturbances attributed to them individually or in combination. While **elevated circulating succinate levels** were initially observed in rodent models of obesity and T2D, the same authors failed to confirm these findings in a small group of patients with T2D³³⁵. However, another study reported that patients with diabetes and hyperglycemia had higher fasting levels of circulating succinate than their normoglycemic peers³³⁹. Our recent observations corroborated these results^{338,340,352}, which showed that plasma succinate levels were higher in people with obesity than in lean individuals, with a comparable increase in patients with T2D and similar BMI. Remarkably, we found that elevated circulating succinate levels are associated with several markers of a **worse metabolic profile**, including insulin, glucose, HbA1c, homeostatic model assessment of insulin resistance, diastolic blood pressure, and triglycerides. After adjusting for confounders, BMI and glucose were the primary determinants of circulating succinate³³⁸, suggesting that both obesity and T2D impact this metabolite in blood. We also found similar observations in patients with T2D and morbid obesity, in whom surgical weight loss intervention decreased circulating succinate levels^{340,352}. Remarkably, we noted that preoperative succinate levels can **predict diabetes remission** following bariatric surgery, enabling the selection of optimal surgical procedures for individual patients regarding T2D remission and potential complications³⁴⁰. Succinate, previously identified as a prognostic marker in critically injured patients³³⁷, cancer patients³⁵⁷, and more recently, critically ill COVID-19 patients^{358,359}, presents novel insights as a significant indicator of metabolic perturbations. As a result, succinate has the potential to offer a more comprehensive understanding of homeostasis as a readily measurable blood-based metabolic biomarker.

Furthermore, our findings indicate that the nutritional modulation of plasma succinate, observed under healthy circumstances, is impaired in individuals with T2D

and morbid obesity; however, this modulation is restored following weight loss³⁵². This pattern parallels the behavior of certain hormones in an obesogenic context, including leptin and GLP-1^{360,361}. However, dietary succinate is not effective in reversing the metabolic consequences of obesity once it is established³⁶², as it might be a succinate-resistant state in this disease²⁷².

Metabolic-associated fatty liver disease (MAFLD) is a pathological condition resulting from fat buildup in the liver, causing various complications such as liver failure, cirrhosis, and even hepatic cancer^{363,364}. MAFLD is a product of metabolic dysfunction and is closely tied to obesity, T2D, and cardiovascular disease³⁶⁵. In this context, succinate has emerged as a crucial player in activating hepatic stellate cells, which are vital in hepatic fibrosis development^{366,367}. Studies conducted on animal models of liver fibrosis and steatohepatitis have indicated a correlation between succinate and disease pathogenesis^{368,369}. However, no translational studies have been performed on humans. Although the evidence is not yet sufficient to conclude a role for succinate in the development of steatosis from a metabolic perspective, elevated levels of liver succinate have been reported in patients at various stages of liver disease, including cirrhosis and MAFLD^{370,371}. Plasma metabolomic analyses of biopsy-proven MAFLD patients showed enrichment of succinate in patients with advanced fibrosis³⁷². More recently, a set of metabolic biomarkers, including succinate, has been identified as being elevated in MAFLD³⁷³.

We recently conducted a cross-sectional study investigating the relationship between plasma succinate levels and **cardiovascular disease** risk in young individuals³⁵⁶. Our findings revealed a significant association between elevated plasma succinate levels and increased visceral white AT mass, triglycerides, C-reactive protein, and diastolic blood pressure. Furthermore, individuals with higher succinate levels exhibited a distinct pro-inflammatory omega-6 signature pattern. These observations suggest that plasma succinate levels could serve as a viable biomarker for assessing the cardiovascular health of young adults, underscoring its emerging utility as a potential marker for inflammatory and metabolic disturbances in younger demographics³⁵⁶.

In summary, analyzing circulating succinate levels in various metabolic diseases, including obesity, T2D, MAFLD, and cardiovascular disease, has shed light on its potential as a valuable metabolic biomarker. The association of elevated succinate levels with worse metabolic profiles and the ability to predict T2D remission after bariatric surgery highlights its clinical relevance. Furthermore, the impaired modulation

of plasma succinate after food ingestion in individuals with T2D and morbid obesity, which is restored after weight loss, emphasizes its dynamic nature and potential role as a metabolite-like hormone. Future research should focus on elucidating the underlying mechanisms of succinate's involvement in metabolic disease pathogenesis and exploring its potential for guiding personalized therapeutic interventions and monitoring treatment outcomes.

1.3.4. The Succinate Receptor 1

The complexity of SUCNR1 can be best interpreted through a foundational understanding of its receptor family. Accordingly, the subsequent section outlines the nature and functionality of GPCRs. Following this, the focus will shift to the signal transduction pathways mediated by SUCNR1 and its discovered roles in metabolic regulation.

1.3.4.1. Structure and Signal Mechanisms of GPCRs

As one of the most extensive and heterogeneous protein families, GPCRs hold a crucial position in cellular communication and signal transduction in response to a wide variety of extracellular cues³⁷⁴. GPCRs play an indispensable role in normal development and physiology, and alterations in these receptors or their transducers can significantly impact disease initiation and progression, including T2D³⁷⁵. In line with this, GPCRs are heavily implicated in pharmacotherapy, as they are largely targeted by currently approved medications despite only covering a small fraction of the entire GPCR repertoire³⁷⁵. Consequently, there is a growing interest in investigating and exploiting these proteins for therapeutic purposes^{374,375}.

Despite their extensive sequence diversity, GPCRs possess a conserved structural architecture comprising a transmembrane domain with seven helices embedded in the cell membrane. These transmembrane helices, critical for signal transduction, are connected by three extracellular loops and three intracellular loops. However, subtle structural distinctions exist among members of different classes, even with their overall structural resemblance^{374,376}.

Canonical GPCR signal transduction relies on receptor-mediated activation of heterotrimeric G proteins, which consist of three subunits: $G\alpha$, $G\beta$, and $G\gamma$ ³⁷⁷. The differential coupling of GPCRs to various **$G\alpha$ proteins** imparts functional diversity and specificity to each GPCR^{377,378}. Despite the vast array of GPCR genes present in the

human genome, only four primary $G\alpha$ -protein families (i.e., G_s , G_i , G_q , and $G_{12/13}$) have been identified based on sequence homology among over 21 human $G\alpha$ isoforms encoded by 16 individual genes³⁷⁶.

In its inactive state, the $G\alpha$ subunit binds to guanosine diphosphate (GDP) and associates with the $G\beta\gamma$ dimer to form the inactive heterotrimer. Upon receptor activation, the GDP-bound heterotrimer engages with the receptor, accelerating GDP dissociation from $G\alpha$, which is the rate-limiting step in G-protein activation. Due to the high cellular GTP concentration, the transient nucleotide-free receptor-G-protein complex quickly binds GTP. This binding causes conformational changes in the $G\alpha$ subunit, resulting in the separation of the $G\alpha$ and $G\beta\gamma$ subunits. Both subunits modulate the activity of various downstream effector proteins^{376,379}.

$G\alpha$ subunits modulate different effectors depending on the specific $G\alpha$ type bound to the receptor, while $G\beta\gamma$ recruits GPCR kinases (GRKs) to the membrane and regulates different signaling responses. The signaling mediated by the distinct $G\alpha$ proteins varies: **G_s** targets AC, enhancing their activity and promoting cAMP production, while **G_i** inhibits AC, reducing cAMP levels. The **G_q** family activates PLC, leading to the generation of IP_3 and DAG. These second messengers initiate downstream signaling pathways, including calcium mobilization and PKC activation. **$G_{12/13}$** proteins activate Rho family GTPases, inducing changes in cytoskeletal organization and cell shape^{374,376,379} (**Figure 10**).

Signal termination and GPCR desensitization ensure that cells can respond appropriately to changing stimuli. The cellular response concludes when the $G\alpha$ subunit hydrolyzes GTP to GDP, utilizing its inherent GTPase activity, and reassociates with $G\beta\gamma$, completing the G-protein activation cycle. One primary mechanism of GPCR desensitization involves phosphorylation by GRKs. Upon activation, GRKs phosphorylate intracellular domains of GPCRs, which subsequently leads to the recruitment of β -arrestins^{380,381}. β -arrestins not only sterically hinder further interaction between GPCRs and their cognate G proteins but also promote GPCR internalization via clathrin-mediated endocytosis. This internalization process ultimately results in the sequestration of the receptors in endosomes, where they can either be dephosphorylated and recycled back to the plasma membrane or targeted for lysosomal degradation³⁸⁰. Additionally, the termination of GPCR signaling can also occur through the actions of regulators of G protein signaling proteins, which function as GTPase-activating proteins to accelerate GTP hydrolysis, thereby inactivating G proteins and decoupling them from the receptor³⁷⁶.

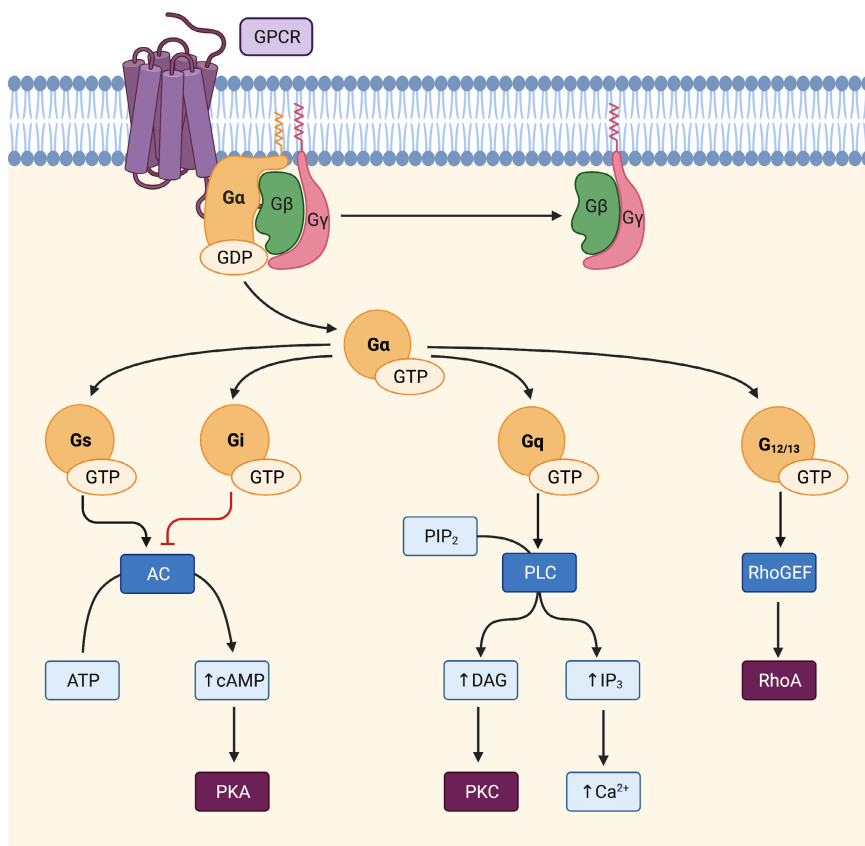


Figure 10. Signaling Pathways Initiated by G-Protein-Coupled Receptor (GPCR) Activation through Gα Proteins. Upon ligand engagement, GPCRs activate different Gα proteins: Gs promotes adenylyl cyclase (AC) activity, elevating cyclic AMP (cAMP) levels, while Gi inhibits AC, reducing cAMP concentrations. Gq activates phospholipase C (PLC), producing inositol trisphosphate (IP₃) and diacylglycerol (DAG). G_{12/13} triggers Rho family GTPases, influencing cell shape and cytoskeletal organization. ATP: adenosine triphosphate; GDP: guanosine diphosphate; GTP: guanosine triphosphate; PIP₂: phosphatidylinositol 4,5-bisphosphate; PKA: protein kinase A; PKC: protein kinase C.

1.3.4.2. Signaling Pathways Mediated by the Succinate Receptor 1

One GPCR of particular interest is the **SUCNR1**, also known as G-protein coupled receptor 91 (GPR91)²⁷⁷. SUCNR1 is predominantly expressed in the kidneys, liver, heart, spleen, and retina^{277,382–384}, and its endogenous ligand is succinate²⁷⁷. Although it has been suggested that SUCNR1 can bind other carboxylic acids, their affinity is much lower than succinate (succinates' EC₅₀ is approximately 20–50 μM) and is unlikely to have physiological relevance²⁷². The gene encoding SUCNR1 is situated on a cluster of GPCRs in human chromosome 3. Among potential receptor PTMs, SUCNR1's extracellular N-terminus contains two N-glycosylation sites and one phosphorylation

site is located in the intracellular C-terminal region. However, the functional role of these PTMs remains unknown²⁷². Upon activation, SUCNR1 triggers different signal transduction pathways, which vary according to the cell type. SUCNR1, like other GPCRs, can undergo desensitization and internalization; however, the processes that control these mechanisms are not known²⁷².

Research has shown that SUCNR1 can couple to different G α proteins, resulting in a variety of downstream signaling cascades and biological effects. In the **kidneys**, where SUCNR1 expression is particularly elevated in the vascular lumen of the juxtaglomerular apparatus (JGA) and the renal tubules^{385,386}, it has been reported to couple to Gi proteins, which activates the renin-angiotensin system, contributing to an increase in blood pressure^{277,387,388}. SUCNR1 is also expressed in the luminal membrane of tubular cells, and succinate can trigger arachidonic acid and prostaglandin release in these cells³⁸⁵. Given that succinate is filtered and reabsorbed in the kidney, SUCNR1 has been proposed as a modulator of this process, as it appears to play a regulatory role in nephron succinate filtration and reabsorption^{327,332}. Moreover, the accumulation of succinate in the kidney and urine of mice with streptozotocin-induced diabetes has been linked to elevated renin release from JGA by a SUCNR1-dependent mechanism³⁸⁹. Furthermore, recent studies have shown that SUCNR1 is also expressed in the luminal membrane of tubular cells in the kidney, where it can sense alterations in local tissue metabolism via accumulating tubular succinate and SUCNR1 signaling³⁸⁷.

In the **heart**, SUCNR1 signaling via Gi and Gq proteins is involved in regulating cardiac hypertrophy and fibrosis. Activation of SUCNR1 in cardiomyocytes results in the activation of PKC and the calcineurin-nuclear factor of activated T-cells (NFAT) pathways. These signaling events contribute to the pathological remodeling of the heart in response to stress and injury^{355,390}.

In the **retina**, SUCNR1 has been implicated in the regulation of vascular development and angiogenesis³⁸³. Activation of SUCNR1 in retinal ganglion cells activates ERK1/2 and JNK signaling pathways, with the subsequent induction of vascular endothelial growth factor production, cyclooxygenase-2 expression, and prostaglandin E2 release^{391,392}.

Remarkably, SUCNR1 has garnered significant attention for its involvement in **inflammatory diseases** and **pathological contexts**^{272,393}. Despite pro-inflammatory macrophages releasing succinate^{394,395}, anti-inflammatory or pro-resolving macrophages predominantly express SUCNR1^{395,396}. Earlier findings showed that SUCNR1

activation enhances IL-1 β secretion via Gi in interferon-primed macrophages³⁹⁴, though a subsequent investigation indicated that macrophages isolated from global *Sucnr1*-deficient mice have a cytokine profile similar to those obtained from control mice³³⁹. Notably, we have recently demonstrated that succinate-SUCNR1 signaling fosters an anti-inflammatory macrophage phenotype. The effect of *Sucnr1* deficiency on adipose tissue macrophages (ATMs) relies on their inflammatory state. Specifically, *Sucnr1*-deficient ATMs exhibit a pro-inflammatory phenotype in subcutaneous AT compared to their wild-type equivalents. In contrast, *Sucnr1*-deficient ATMs in visceral AT reveal a subdued inflammatory condition³⁹⁵. Moreover, SUCNR1 signaling via Gq in M2 macrophages activates the PLC-mediated pathway, which has been shown to promote anti-inflammatory responses by upregulating the expression of anti-inflammatory cytokines³⁹⁷.

These observations emphasize the intricacy of tampering with the succinate-SUCNR1 pathway in macrophages, the consequences of which will likely be context-specific. Nevertheless, we have proposed that succinate is generated as part of the inflammatory response to encourage a strong reaction during the acute inflammatory phase while simultaneously serving as a feedback signal via the promotion of pro-resolving macrophages in a SUCNR1-dependent manner²⁷². In line with the hypothesis of succinate as a 'resolving' agent, macrophage-derived succinate has recently been identified as a crucial element in the anti-inflammatory effects of neural stem cells during chronic neuroinflammation. In this situation, succinate release by pro-inflammatory macrophages signals through SUCNR1 in neural stem cells, leading to the secretion of prostaglandin E2 and extracellular succinate scavenging, which ultimately resolves inflammation³⁹⁸. Given that immune system disturbances also contribute to metabolic disorders, it is unsurprising that SUCNR1 plays a role in metabolic pathophysiology. In fact, numerous studies have documented a tight connection between nutrient surplus and immune system activation in metabolic organs, which directly correlates with insulin resistance and T2D³⁹⁹.

Collectively, these findings emphasize the relevance of SUCNR1 in the context of pathological conditions and underscored the potential therapeutic implications of targeting this receptor in the treatment of various pathologies associated with inflammation.

1.3.5. Current Knowledge on the Succinate-SUCNR1 Axis in Metabolic Regulation

Until recently, the prevailing assumption held that SUCNR1 remained quiescent in healthy tissues, only springing into action under pathological conditions concomitant with hypoxia and tissue damage, coinciding with an upsurge in extracellular succinate. In tandem with this view, SUCNR1 signaling had predominantly been linked to pathophysiology, encompassing an array of diabetes-related comorbidities such as hypertension³⁵⁵, renal^{385,387,389}, and retinal complications^{383,391}; MAFLD^{400,401}, inflammatory bowel disease³⁴⁴, age-related macular degeneration⁴⁰², rheumatoid arthritis^{394,403,404}, and certain neoplasms^{405–407}. Nevertheless, an expanding body of evidence now posits that SUCNR1 serves as a linchpin in metabolic regulation and physiological states, challenging the previous paradigm.

In a landmark study, McCreath and colleagues uncovered a significant role of SUCNR1 in metabolism, as it was revealed that SUCNR1 plays a role in regulating **adipose mass** and **glucose homeostasis**⁴⁰⁸. By developing a **whole-body SUCNR1 knockout (KO)** mouse, the researchers discovered that succinate receptor deficiency resulted in contrasting effects on metabolism and overall body weight. Interestingly, these disparities were found to be dependent on the type of feeding challenge. When fed a normal chow diet, SUCNR1-KO mice demonstrated reduced WAT mass, smaller adipocytes, increased energy expenditure, and enhanced glucose buffering compared to control mice. It was initially hypothesized that decreased SUCNR1 expression would result in diminished expression of genes involved in adipocyte differentiation. However, the deletion of SUCNR1 did not impact adipogenesis; instead, it led to decreased lipid accumulation –consistent with previous observations reporting that SUCNR1 inhibits lipolysis in adipocytes³⁸⁴– and reduced adipocyte size. The authors noted that the antilipolytic effects of succinate-SUCNR1 signaling in adipocytes could be a reasonable explanation for the moderate lean phenotype of whole-body SUCNR1-KO mice in basal conditions. In stark contrast, under a high-fat diet (HFD) feeding, SUCNR1-KO mice exhibited a poorer metabolic profile compared to control mice, characterized by worsened glucose tolerance accompanied by decreased insulin secretion. Intriguingly, pancreatic islet architecture, in terms of islet area and β -cell mass, remained unaltered, and the total insulin content in islets was unchanged between SUCNR1-KO and control mice under HFD. This study not only expanded our understanding of the metabolic implications of SUCNR1 using a powerful research tool (i.e., a transgenic mouse model with whole-body SUCNR1

deletion) but also highlighted the importance of diet in determining the resulting observed disparities.

Furthermore, the study underscored the **pleiotropic** and **complex phenotype** arising from whole-body SUCNR1 deletion, emphasizing the need for additional research to elucidate the specific role of SUCNR1 in each tissue and cell-specific context. As SUCNR1 is broadly expressed across tissues, including those metabolically active^{277,384,409}, and energy homeostasis involves tissue crosstalk, identifying the exact role of extracellular succinate as a signaling molecule requires targeted genetic approaches that focus on specific cell types involved in SUCNR1 signaling. Following this perspective, certain studies have delved into the influences of SUCNR1 activity across diverse metabolic tissues.

Specifically in **AT**, SUCNR1 was found to be highly abundant in white AT (WAT) and purified adipocytes^{384,408}. It was observed that succinate inhibits lipolysis in a pertussis toxin-dependent manner, implicating Gi protein-coupled signaling in the antilipolytic effects of the succinate-SUCNR1 axis³⁸⁴. Recent findings suggested that the core antilipolytic effect of succinate arises from its export from mitochondria, which may influence circulating nonesterified fatty acids and liver steatosis⁴¹⁰. It is well-established that AT lipolysis is tightly regulated by hormones secreted based on nutritional demands. For instance, insulin reduces lipolytic activity in WAT during postprandial states due to its antilipolytic action⁴¹¹. The recent observation that circulating succinate levels increase during the fasted-to-fed transition³⁵² may align with its antilipolytic function.

Notably, we have recently described the succinate-SUCNR1 axis as a novel regulator of leptin production in adipocytes, a crucial factor in maintaining energy homeostasis⁴¹². Accordingly, SUCNR1 signaling modulates leptin expression through an AMPK-JNK-basic helix-loop-helix ARNT-like 1 (BMAL1)-CCAAT-enhancer-binding protein alpha (C/EBPα)-dependent pathway and governs the circadian clock in adipocytes. Furthermore, the metabolic impact of SUCNR1 deficiency in adipocytes is dependent on nutritional status. ‘Obese’ adipocytes exhibit increased SUCNR1 expression and heightened sensitivity to succinate-induced leptin release. As a result, we have observed a correlation between obesity and the overstimulation of succinate-SUCNR1 signaling in adipocytes, which subsequently amplifies leptin production and is associated with hyperleptinemia in human subjects with obesity. Thus, this study positions the succinate-SUCNR1 axis as an integral component of the **metabolite-sensing machinery**, allowing adipocytes to orchestrate metabolic

reactions in response to nutrient consumption. Consequently, fluctuations in circulating succinate during the transition from fasting to feeding may serve a physiological purpose not only in AT by modulating lipolysis but also in leptin regulation *via* SUCNR1 through a complex mechanism involving the interplay of the circadian clock, further highlighting the intricacies of this system⁴¹². Moreover, the detrimental effects of *Sucnr1* inactivation in myeloid cells, leading to inflammation and glucose intolerance, exacerbating diet-induced obesity, occur mainly to detrimental effects in the AT³⁹⁵.

In contrast to AT, SUCNR1 is minimally expressed in BAT, and there is no assigned function for it in this compartment yet^{272,408}. However, as previously described in this chapter, succinate has been identified as an intracellular signaling molecule regulating BAT physiology thanks to its thermogenic properties³³⁶. These same authors recently demonstrated that brown adipocytes control extracellular succinate levels in the liver in an obesogenic environment *via* an UCP1-succinate-SUCNR1 pathway, a new mechanism that could be employed to improve liver inflammation caused by SUCNR1⁴¹³.

Moreover, the axis is also involved in glucose metabolism at the **intestinal level**, where dietary succinate has been shown to improve glucose tolerance in mice through intestinal gluconeogenesis³⁴⁵. Although the existence of SUCNR1 in the intestine is still a matter of debate, it has been suggested that tuft cells might express it to sense succinate secreted by pathogens and activate mucosal type 2 immunity^{414,415}.

The role of succinate in **skeletal muscle** physiology has been investigated for several decades. While early studies showed that circulating succinate levels increase during anaerobic exercise³⁴⁸, it was not until recently that the succinate-SUCNR1 axis was identified as a key regulator of skeletal muscle fiber remodeling. In line with this, a study further investigated the role of succinate in skeletal muscle physiology and confirmed that both human and mouse muscle secretes succinate during exercise in an MCT-1-dependent manner, which is highly sensitive to changes in pH. According to this study, succinate released from exercising muscle mediates muscle adaptation in non-myofibrillar resident cells, such as endothelial and satellite cell populations, *via* SUCNR1³²⁶.

In summary, these findings align with previous research on the significance of succinate in metabolic regulation, further emphasizing the crucial role of the succinate-SUCNR1 axis in maintaining metabolic homeostasis and regulating physio-

logical processes such as blood pressure, insulin secretion, adipose tissue lipolysis, glucose metabolism, and skeletal muscle adaptation²⁷². Although SUCNR1 expression is low in some tissues, its involvement in several key physiological processes highlights its potential as a therapeutic target for various diseases. It is essential to note, however, that not all aspects of SUCNR1 function in metabolic regulation have been explored, and further research is required to fully elucidate its diverse roles and to understand the mechanisms of action and potential implications of targeting the axis in these contexts.



Hypothesis and objectives

2



UNIVERSITAT ROVIRA I VIRGILI

THE SUCCINATE-SUCNR1 AXIS AND ITS IMPACT ON BETA-CELL FUNCTION: AN INTEGRATIVE STUDY
UNCOVERING NOVEL INSIGHTS INTO INSULIN SECRETION REGULATION AND DIABETES PATHOGENESIS

Joan-Martí Sabadell i Basallote

2.1. Research Gap and Motivation

Pancreatic β -cell function stands as a cornerstone in upholding blood glucose homeostasis, with any disturbance in insulin secretion largely influencing the development of T2D¹³⁸. Despite considerable advances in understanding the key players and mechanisms regulating β -cell physiology, gaps remain in our knowledge of how these pathways interact and are involved in T2D pathogenesis. Moreover, the alarming increase in T2D prevalence¹⁵ highlights the importance of addressing these uncertainties to enhance our comprehension of the underlying mechanisms and facilitate forging innovative therapeutic strategies.

As previously discussed, insulin secretion is a sophisticated process overseen by a multitude of pathways and factors, including the well-known K_{ATP} -dependent triggering pathway and GPCR-mediated neurohormonal amplifying pathways^{35,69}. In line with this, GPCRs –an evolving research area– have demonstrated their considerable influence on insulin release modulation with recognized GPCRs, such as GLP-1R and FFAR1, already acknowledged as important potentiators of insulin secretion^{11,416–418}. Nevertheless, other GPCR-based mechanisms controlling insulin secretion await investigation. Therefore, examining novel GPCRs within the framework of β -cell function holds great promise to enhance our understanding of the intricacies of insulin secretion and associated factors that may drive T2D progression.

Exploring the factors that influence β -cell function has broad implications, extending beyond T2D to include prediabetes, a state which, if not mitigated, can evolve into T2D^{138,146}. Notably, our current grasp of compensatory hyperinsulinemia mechanisms in prediabetes and their potential role in T2D progression is limited, emphasizing the importance of investigating new targets and processes associated with β -cell adaptation and dysfunction^{138,166}.

These knowledge gaps carry particular significance when considering the current treatments for T2D, which predominantly aim at increasing insulin sensitivity or counteract the diminished insulin secretion capacity of β -cells^{98,138}. However, the efficacy of these treatments is limited, and they often fail to prevent disease progression or the development of complications^{138,255,419}. This highlights the need to identify novel pathways governing β -cell function and insulin secretion compensation to devise more effective treatment options to halt or reverse T2D progression.

2.2. Study of the Succinate-SUCNR1 Axis in the β -Cell as a Potential Regulator of Insulin Secretion

The role of the succinate-SUCNR1 axis in metabolic regulation has gained increasing attention due to its potential implications in the context of T2D pathogenesis. Previous research has shown that the TCA cycle substrate succinate not only participates in energy metabolism but also functions as a signaling molecule, accumulating in the cytosol and extracellular space^{272,342}. As a result, succinate engages with its receptor, SUCNR1²⁷⁷, which is reportedly expressed in the pancreas and islets^{384,409,417}, indicating a potential yet unexplored role of the succinate-SUCNR1 axis in islet physiology. In the context of the β -cell function, it has long been established that cell-permeable succinate esters are potent insulin secretagogues^{315,318}, intracellular succinate potentially acts as a stimulus-coupling secondary signal for proinsulin biosynthesis^{316,321,322}, and intracellular succinate is closely associated with 2nd phase insulin secretion³²³. Although significant progress has been made in understanding the intracellular functions of succinate in β -cell physiology, its extracellular roles in glucose-mediated insulin secretion have not been investigated.

Historically, it was posited that SUCNR1 remained inactive in healthy tissues, becoming active only under pathological conditions associated with hypoxia and tissue damage, which coincided with increased extracellular succinate levels. SUCNR1 signaling was thus primarily linked to pathophysiological processes, including numerous diabetes-related comorbidities. However, recent observations revealed that succinate transiently rises during physiological states, suggesting that succinate serves as a systemic metabolic signal during physiological processes through SUCNR1²⁷². Moreover, we demonstrated that dynamic succinate regulation occurs during the fasted-to-fed transition, which is potentiated when nutrients are sensed by the intestine but is disturbed in morbid obese patients with T2D³⁵². Therefore, the initial view of SUCNR1 as an exclusive sensor for local damage is being reconsidered in light of recent observations of succinate as a systemic metabolic-signal regulated in postprandial states.

SUCNR1 has been shown to signal preferentially through cAMP- or Ca^{2+} -dependent pathways^{272,393}, both of which are well-established regulators of insulin secretion in β -cells^{11,420}. Notably, global *Sucnr1*-knockout mice fed a high-fat diet exhibit impaired insulin secretion and glucose intolerance without significant changes in β -cell mass, indicating a potential role for SUCNR1 in regulating β -cell function⁴⁰⁸. This study exposed the multifaceted and intricate phenotype resulting from global SUCNR1

ablation, emphasizing the need for further research to elucidate the specific role of SUCNR1 in different tissues and cell-specific contexts. Consequently, recent investigations have explored the effects of SUCNR1 activity in specific metabolic tissues, uncovering its critical role in regulating physiological processes^{272,326,395,412}.

Considering these discoveries, the **hypothesis of this thesis** is that the succinate-SUCNR1 axis may serve as a glucose-dependent, novel amplifying transduction pathway enhancing insulin release, functioning as an incretin-like metabolite, which may significantly potentiate insulin secretion during hyperglycemia. Understanding the role of the succinate-SUCNR1 axis in regulating insulin secretion and the development of T2D could offer new opportunities for designing targeted therapies, such as specific modulators of the succinate-SUCNR1 pathway. To address the central hypothesis of this thesis, the following **objectives** will be pursued:

1. Characterize the expression of SUCNR1 in pancreatic islets and β -cells and how it is modulated according to metabolic states.
2. Elucidate the potential effects of the activation of the succinate-SUCNR1 axis in β -cells.
3. Decipher the underlying mechanisms regulating SUCNR1 transduction in β -cells.
4. Determine the contribution of SUCNR1 in *in vivo* insulin secretion in basal and pathological states.
5. Study succinate dynamics in a cohort of prediabetic human subjects and its potential association with insulin secretion potentiation.

Therefore, this study aims to investigate the impact of the succinate-SUCNR1 axis on β -cell function by employing a comprehensive and integrative translational approach, including the use of *in vitro* approaches, a unique pancreatic β -cell-specific knockout mouse model, and the study of a prediabetic human cohort. Through a better understanding of this novel pathway, this research has the potential to significantly contribute to developing more effective and personalized strategies to address the global burden of T2D and related metabolic disorders.

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THE SUCCINATE-SUCNR1 AXIS AND ITS IMPACT ON BETA-CELL FUNCTION: AN INTEGRATIVE STUDY
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3

Materials and methods

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3.1. Human Studies

3.1.1. Human Cohort for the Functional Study

The conduct of the study protocols was in accordance with the ethical principles outlined in the Declaration of Helsinki and received authorization from the Institut d'Investigació Sanitària Pere Virgili (IISPV) Ethics Research Committee (reference number 204/2017). Prior to their participation, all subjects were given a thorough explanation of the study protocol and provided written informed consent. The study was conducted at the Unit of Functional Studies, IISPV, Hospital Universitari Joan XXIII in Tarragona (Spain). As part of a screening protocol for obesity in the general population, healthy asymptomatic subjects were selected from those who visited the Endocrinology Service. The main inclusion criteria were as follows: age between 18-65 years, BMI < 35 kg·m⁻²; absence of underlying pathologies (cancer, liver disease, kidney disease, or systemic inflammation) on physical examination, diagnostic tests other than those associated with an excess of weight; and signing the protocol-informed consent. Additionally, inclusion criteria required a level of HbA1c < 6.5% and a fasting glucose level < 126 mg·dl⁻¹ on two separate occasions. The main exclusion criteria were pregnancy and lactation; vegetarianism, veganism, or eating disorders; chronic treatment with anti-inflammatory agents, antibiotics, or cortisol; and major psychiatric antecedents, alcoholism, or drug abuse.

After the initial triage, thirty subjects were selected to be submitted to an oral glucose tolerance test (OGTT) with 75 g of glucose. Individuals were classified according to the ADA criteria⁴²¹ as normal glucose tolerant (NGT; fasting glucose levels ≤ 110 mg·dl⁻¹ and a normal OGTT response) or non-NGT (fasting glucose levels ≥ 110 and < 126 mg·dl⁻¹, or impaired glucose tolerance or diabetes during an OGTT following the ADA criteria), which are also referred to as prediabetic patients (the clinical information is summarized in **Results Table 4**, page 123).

3.1.2. Assessment of β -Cell Function and Succinate Dynamics in Humans

3.1.2.1. Procedures and Clinical Determinations

All participants underwent two procedures on separate occasions. First, we applied a 3-hour OGTT and, subsequently, a 3-hour intravenous isoglycemic glucose infu-

sion (IIGI), reproducing the plasma glucose time curve recorded during the OGTT using an *ad hoc* algorithm. Plasma glucose was determined every ten minutes during both procedures, whereas insulin, C-peptide, GLP-1, and succinate were determined in plasma samples at -30, 0, 10, 20, 30, 60, 90, 120, 150, and 180 minutes. Body composition was analyzed by electric bioimpedance (Tanita Europe BV; Amsterdam, The Netherlands). Plasma lipid, hepatic and renal profiles were determined by standard enzymatic methods. Plasma glucose was determined by the glucose oxidase method (GM-9; Analox, London, UK). Plasma insulin and C-peptide levels were determined by an immunochemiluminometric assay (ADVIA Centaur; Siemens Healthcare, Erlangen, Germany). The above determinations were carried out in the Clinical Laboratory of Hospital Universitari Joan XXIII. Total plasma GLP-1 levels were examined by enzyme-linked immunosorbent assay (ELISA; EZGLP1T-36 K; Merck, Darmstadt, Germany) following the manufacturer's instructions. Plasma succinate was determined in plasma filtrates (10 kDa) using a fluorometric assay (EnzyChrom Succinate Assay Kit; BioAssay Systems, Hayward, CA).

3.1.2.2. Mathematical Modeling

Insulin sensitivity was estimated from the plasma glucose and insulin responses to oral glucose using the OGTT-derived insulin sensitivity index (OGIS)²¹⁴. *In vivo* insulin secretion, β -cell function parameters, and the incretin effect were calculated by simultaneous mathematical modeling analysis of the IIGI and the OGTT data²¹³ performed in a custom MATLAB-based algorithm²¹³ (**Figure 11**). Parameter estimations were obtained by a nonlinear least-squares fitting approach through the *lsqnonlin* function. This model reconstructs insulin secretion from plasma C-peptide using an established model of C-peptide kinetics⁴²², and it extends a widely used model for the analysis of the OGTT⁴²³.

In the IIGI test, the correlation between the amount of glucose present and the release of insulin remains consistent with the traditional model (**Equation 1**)^{423,424}:

$$S_{IIGI}(t) = P_{GLU}(t) \cdot f(G_{IIGI}(t)) + rs_{IIGI} \cdot \frac{d^+G_{IIGI}(t)}{dt} \quad (1)$$

In the context of the IIGI test, various factors contribute to insulin secretion (denoted as $S_{IIGI}(t)$). These factors include the rate sensitivity (rs_{IIGI}), the positive derivative of glucose concentration ($d^+G_{IIGI}(t)/dt$) with respect to time (i.e., $dG_{IIGI}(t)/dt$) when $dG_{IIGI}(t)/dt > 0$ and otherwise it is zero), $f(G)$ as the dose-response function that

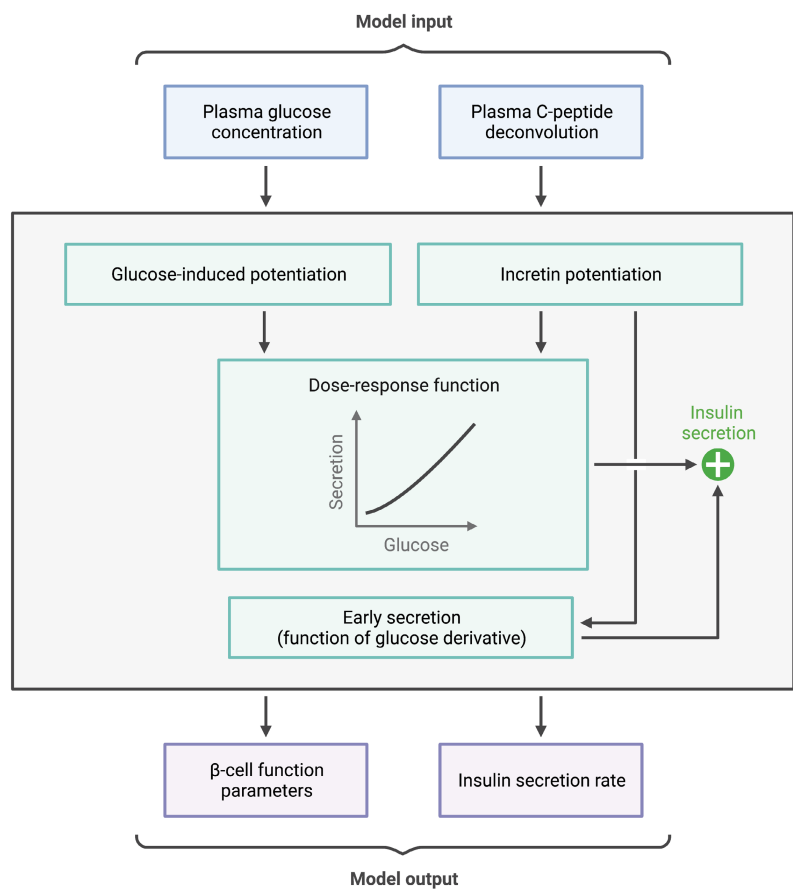


Figure 11. Schematic Representation of the Integrated Model of Insulin Secretion and Potentiation Effect. This diagram depicts the mathematical model employed to assess the impact of the potentiation effect on insulin secretion and β -cell function. This model incorporates data from oral glucose tolerance tests (OGTTs) and subsequent isoglycemic intravenous glucose infusions (IIGIs). The diagram presents both the inputs and outputs along with their respective parameters, thereby providing a summarized view of the system dynamics. Adapted from Dr. Tura’s work^{213,215}.

connects insulin secretion to glucose concentration, and $P_{GLU}(t)$ is the potentiation factor that modulates the dose-response. The potentiation factor accounts for the fact that insulin secretion at a given glucose level may differ during the test. $P_{GLU}(t)$ is assumed to average to one during the IIGI test. In intravenous tests, this modulation has been classically attributed to exposure to relative hyperglycemia, and thus the term $P_{GLU}(t)$ is referred to as glucose-induced potentiation.

During the OGTT, insulin secretion (represented as $S_{OGTT}(t)$) follows a similar pattern as in the IIGI test but with an additional potentiation term called $P_{INCR}(t)$, referred to as incretin potentiation. This incretin potentiation term amplifies the effect of the dose-response relationship. Unlike the $P_{GLU}(t)$ term in the IIGI test, $P_{INCR}(t)$ is not re-

stricted to averaging to one. Additionally, the rate sensitivity parameter differs from the IIGI test. Therefore, the expression for insulin secretion during the OGTT is as follows (**Equation 2**):

$$S_{OGTT}(t) = P_{INCR}(t) \cdot P_{GLU}(t) \cdot f(G_{OGTT}(t)) + rs_{OGTT} \cdot \frac{d^+ G_{OGTT}(t)}{dt} \quad (2)$$

Hence, the extent of the incretin effect is measured by the time-dependent incretin potentiation $P_{INCR}(t)$ and the OGTT-specific rate sensitivity rs_{OGTT} . The dose-response function $f(G)$ and the glucose-induced potentiation $P_{GLU}(t)$ remain unchanged from the IIGI test (as defined in **Equation 1**).

Moreover, the model incorporates equations for fitting the glucose concentration in both the IIGI test ($G_{IIGI}(t)$) and the OGTT ($G_{OGTT}(t)$). These equations serve the purpose of smoothing and interpolating the measured glucose values, similar to the approach used in the previous model, where $k = 0.012 \text{ min}^{-1}$ is an assigned constant, and $R_{IIGI}(t)$ and $R_{OGTT}(t)$ are functions of time (**Equations 3 and 4**):

$$\frac{dG_{IIGI}(t)}{dt} = -k \cdot G_{IIGI}(t) + R_{IIGI}(t) \quad (3)$$

$$\frac{dG_{OGTT}(t)}{dt} = -k \cdot G_{OGTT}(t) + R_{OGTT}(t) \quad (4)$$

Additionally, the model incorporates the two-exponential model of C-peptide kinetics previously proposed⁴²², which allows for the calculation of C-peptide concentration based on the insulin secretion obtained from **Equations 1 and 2**.

The parameter estimation process involves determining the four parameters of the β -cell dose-response function $f(G)$, the two rate sensitivity parameters (rs_{IIGI} and rs_{OGTT}), and four time-dependent functions representing the potentiation factors ($P_{GLU}(t)$ and $P_{INCR}(t)$), as well as the glucose smoothing terms, $R_{IIGI}(t)$ and $R_{OGTT}(t)$. To achieve this, the functions $R(t)$ and $P(t)$ are represented as piecewise linear functions over a 5-minute time grid, employing the regularized least-squares approach using the *lsqnonlin* algorithm, which was employed for parameter estimation. These regularization terms ensure the smoothness of $R(t)$ and $P(t)$ to achieve a more accurate and reliable model fit²¹³.

In the employed mathematical model, two key parameters were derived from the potentiation factors: the ratio of glucose-induced potentiation between the end of

the IIGI test and the baseline, providing insights into the $P_{\text{GLU}}(t)$ excursion and the mean incretin potentiation, calculated as the integral of $P_{\text{INCR}}(t)$ over time divided by the test duration, which represents the overall incretin effect. In addition, the classical total incretin effect was assessed by calculating the ratio of the total insulin secretion during the OGTT to that during the corresponding IIGI test⁴²⁵.

In summary, the model depicts the relationship between insulin secretion and glucose concentration during the IIGI as a quasi-linear function (i.e., the glucose dose-response). Additionally, it describes a time-dependent factor modulates this response during the test, which averages one. This time-dependent factor, denoted as glucose-induced potentiation, accounts for potentiation phenomena that typically prompt glucose-dependent insulin secretion higher at the end of the test compared to the beginning. The model also includes a term describing early secretion related to first-phase insulin release. The same terms define insulin secretion during the OGTT. However, the dose-response is multiplied by another time-dependent factor, typically greater than one, which accounts for the effect of incretin hormones on insulin secretion. This time-dependent factor is denoted as incretin potentiation, and its area under the curve (AUC) is a measure of the total incretin effect.

To study a potential relationship between the insulin secretion potentiation during the IIGI and succinate dynamics, we used the potentiation ratio between 2 hours and the baseline, calculated as the integral of the potentiation over time divided by the total time assessed (which represents the overall incretin effect), and the corresponding 2-hour incremental succinate AUC mean.

3.1.3. Human Islets

Ten adult cadaveric organ donors (available clinical and anthropometric data summarized in **Table 1**) provided pancreatic islets for the gene expression analysis. The use of human islets was approved by the local Ethics Committee of Hospital Universitari de Bellvitge (approval PR239/13), and signed consent was obtained from donor relatives. The tissues were processed at Hospital Universitari de Bellvitge and Institut d'Investigació Biomèdica de Bellvitge (L'Hospitalet de Llobregat, Spain) and islets were obtained using Ricordi's method⁴²⁶ with slight modifications⁴²⁷, through collagenase digestion (Collagenase NB 1 Premium Grade with Neutral Protease NB; Serva Electrophoresis GmbH, Heidelberg, Germany). A refrigerated COBE 2991 cell processor (COBE BCT, Lakewood, CO) with a continuous density gradient was employed to ensure purification. The yield of islets was assessed using dithizone stain-

ing (Sigma-Aldrich, Saint Louis, MO) in duplicate, where a standard islet diameter of 150 µm represented one islet equivalent (IEQ).

Table 1. Characteristic of the Islet Donors for the Gene Expression Analysis

	Donor ID	Gender	Age (years)	BMI (kg·m ⁻²)	HbA1c (%)	Islet purity (%)
Healthy	94/14	Female	32	21.8	n/a	90
	100/15	Male	41	24.7	n/a	80
	101/15	Female	51	22.4	n/a	90
	106/15	Male	80	20.8	n/a	85
	109/16	Female	61	20.6	5.2	80
	119/17	Male	51	29.4	n/a	85
Obesity	134/19	Male	48	29.3	4.8	90
	97/14	Male	54	35.8	n/a	95
	104/15	Male	62	31.3	5.4	85
	110/16	Male	71	30.5	5.2	85

BMI: body mass index; HbA1c: glycated hemoglobin.

Following purification, islets were immediately placed in 25 cm² culture flasks (Sarstedt AG & Co, Nümbrecht, Germany) and cultured in a free-floating manner. The used culture medium was CMRL1066 medium with 5.6 mM glucose (Corning, New York, NY), supplemented with 2 mM L-glutamine (Life Technologies, Grand Island, NY, USA), 10 mM HEPES (Biological Industries, Beit Haemek, Israel), 40 µg·ml⁻¹ gentamycin (Laboratorios Normon, Madrid, Spain), 0.25 µg·ml⁻¹ amphotericin B (Thermo Fisher Scientific, Waltham, MA), 20 µg·ml⁻¹ ciprofloxacin (Fresenius-Kabi, Bad Homburg, Germany), 10 mM nicotinamide (Sigma-Aldrich), and 0.5% human serum albumin (Grifols, Barcelona, Spain). Islets were incubated in a humidified incubator at 37°C and 5% CO₂ for 3-4 days. To maintain optimal conditions, the culture media was replaced every 2 days.

To determine the viability of islet cells, they were cultured in batches of 2800 IEQs within untreated flasks. After 1 and 3 days of culture, the viability of islet cells was evaluated using acridine orange/propidium iodide staining (Sigma-Aldrich). For this purpose, a 50-islet sample was transferred to a 35 mm Petri dish (Greiner Bio-One, Frickenhausen, Germany) containing Dulbecco's Phosphate-Buffered Saline (DPBS). Subsequently, acridine orange and propidium iodide stock solutions were added, achieving a final concentration of 0.67 µM and 75 µM, respectively. Viable cells were identified as acridine-positive cells (green), while dead cells were iden-

tified as propidium iodide-positive cells (red). The viability of islet cells was quantified as the percentage of viable cells compared to the total number of cells (both viable and nonviable) within the islets.

Human islets for protein expression assays (available clinical and anthropometric data provided in **Table 2**) were obtained from the affiliated institutions to the Integrated Islet Distribution Program⁴²⁸ and Prodo Laboratories (Aliso Viejo, CA, USA). All studies and protocols used were approved by the Joslin Diabetes Center’s Committee on Human Studies (approval CHS#5-05). These islets were processed similarly, with slight modifications^{429,430}. Upon receipt, human islets were centrifuged, washed, transferred to Petri dishes (2000–3000 IEQs/dish), and cultured with fresh Miami Media #1A (Corning) overnight at 37 °C and 5% CO₂.

Table 2. Characterisitcs of the Islet Donors for the Protein Levels Analysis

	Donor ID	Gender	Age (years)	BMI (kg·m ⁻²)	HbA1c (%)	Islet purity (%)
Healthy	HP-17031-01	Female	64	24.6	5.4	95
	UNOS ADJV268	Male	39	23.7	n/a	90
	UNOS ADIY035	Male	15	24.6	n/a	80
	HP-17036-01	Male	59	26.9	5.8	90
	HP-20199-01	Male	21	27.3	5.1	90
	MGH (n/a)	Male	38	28.3	n/a	90
Obesity	RRID 12292085	Male	20	35.8	n/a	95
	UNOS ACEY097	Male	34	31.2	5.1	85
	HP-20206-01	Male	22	31.9	5.4	85
Type 2 diabetes	UNOS ADHF167	Female	52	39.9	7.4	95
	HP-19078-T2D	Female	66	30.3	6.5	85
	HP-19171-T2D	Female	66	29.2	7.2	85
	RRID 12597653	Female	42	40.3	9.5	80
	HP-19131-T2D	Male	58	32.7	6.7	85
	HP-18320-T2D	Male	61	27.4	7.1	85
	UNOS ADAHX156	Male	57	34.6	10.4	98

BMI: body mass index; HbA1c: glycated hemoglobin.

3.2. Animals Studies

All animal studies conformed to the “Animal Research: Reporting of *In Vivo* Experiments” (ARRIVE) guidelines and were carried out under the supervision and approval of the relevant local ethics committees, primarily the Animal Welfare and Governmental Ethics Committee of the Universitat Rovira i Virgili (project reference number 10970). All experimental procedures involving animals conformed to the European Union Directive 2010/63/EU and the European Commission Recommendation 2007/526/EC on the protection of animals used for experimental and other scientific purposes, enacted under the Spanish Royal Decrees 53/2013 and 118/2021.

All mice were housed in the Animal Facility of the Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili. Housing conditions consisted of automated 12-h light-dark cycles at 20–22 °C, 40–60% relative humidity, and *ad libitum* access to normal chow diet (NCD) (3.1 kcal% fat; SAFE Diets, A04) or high fat diet (HFD) (60 kcal% fat; Research Diets Inc., D12492), as indicated. HFD availability to mice started at 8 weeks of age until the study endpoint. Male mice were weighed prior to any experimental procedure. At the end of the study, mice were fasted for 16 h and then euthanized by cervical dislocation. Tissues were collected and immediately snap-frozen in liquid N₂ for gene and protein expression analyses or fixed in 4% formaldehyde for histological studies.

3.2.1. Wild-Type Male Wistar Rats

Male wild-type (WT) Wistar rats (Charles River Laboratories, Wilmington, MA) were housed and used according to the guidelines of the Belgian Regulations for Animal Care, with the approval of the local ethical committee at the University Miguel Hernández. Upon arrival, male Wistar rats (Charles River Laboratories) were carefully transferred from their shipping containers to the housing facilities. Rats were housed in the well-regulated environment of the Animal Facility of Miguel Hernández University (Elx, Spain). This facility ensured optimal conditions for rat housing, including a consistent temperature of 20–24 °C, relative humidity of 30–70%, and a 12-hour light/dark cycle. Rats were given *ad libitum* access to a standard rat diet (provided by the institution) and clean, fresh water.

3.2.2. Islet Isolation, FACS Purification, and *Ex Vivo* Culture of Rat α - and β -Cells

3.2.2.1. Rat Islet Isolation

Rat islets were isolated by collagenase digestion and subsequently handpicked. Islets were dissociated into single cells by mechanical and enzymatic dispersion using trypsin (1 mg·ml⁻¹; Sigma-Aldrich) and DNase I (1 mg·ml⁻¹; Roche, Basel, Switzerland) for 5 min at 31 °C under agitation. Dissociated cells were resuspended in Earle's medium (0.79 mM NaH₂PO₄, 0.8 mM MgSO₄, 2.4 mM CaCl₂, 115 mM NaCl, 5.4 mM KCl) containing 2.8 mM glucose.

3.2.2.2. FACS Separation of Rat Islet Cells

Fluorescence-activated cell sorting (FACS) of β - and α -cells was performed using a FACSaria III cell sorter (BD Biosciences, Franklin Lakes, NJ), following published methodology⁴³¹. The percentage of viability was determined after incubation with the DNA-binding dyes propidium iodide (5 μ g·ml⁻¹; Sigma-Aldrich) and Hoechst 33342 (5 μ g·ml⁻¹; Sigma-Aldrich). A minimum of 600 cells were counted in each experimental condition. Two independent observers evaluated viability, one of them blinded to sample identity. The agreement between observers was required to be higher than 90%⁴³¹.

3.2.2.3. Culture and Purity Assessment of *Ex Vivo* Rat Islet Cells

After sorting, purified β -cells were cultured in Ham's F-10 medium containing 10 mM glucose, 2 mM GlutaMAX (Thermo Fisher Scientific), 0.5% bovine serum albumin (BSA) fraction V (Sigma-Aldrich), 50 μ M 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 50 units·ml⁻¹ penicillin, 50 μ g·ml⁻¹ streptomycin (Thermo Fisher Scientific) and 5% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific); α -cells were cultured in the same medium but including 6.1 mM glucose and 10% FBS. The purity of α - and β -cell preparations was evaluated by immunofluorescence and was calculated as a percentage of positive cells for each cell type⁴³². α - and β -cells were immunostained with mouse monoclonal anti-insulin or anti-glucagon antibodies (both from Sigma-Aldrich) for 1 h, followed by incubation with a rabbit anti-mouse secondary antibody conjugated with Alexa Fluor 488 (A32723) or Alexa Fluor 568 (A11031) from Thermo Fisher Scientific.

3.2.3. Wild Type Mice

Male WT C57BL/6 mice (Charles River Laboratories) are largely used as model organisms due to their robust health, extensive background data, and the availability of congenic strains, making them suitable for a wide array of research, including studies of diet-induced obesity and as a genetic background for the generation of transgenic mice⁴³³. Upon arrival at the animal facility, these mice were gently transferred from the transport containers to the housing units, minimizing stress and potential injury.

3.2.4. Diabetic *db/db* Mice

Male diabetic *db/db* mice were sourced from Charles River Laboratories. These mice are particularly relevant models for studying T2D and related metabolic disorders due to their spontaneous mutation in the leptin receptor gene (*Lepr^{db/db}*), which results in obesity, hyperglycemia, and insulin resistance, thus mirroring the pathophysiological features of human T2D⁴³⁴. Upon their arrival at the designated animal facility, *db/db* mice were carefully transitioned from their transport containers to their new housing facilities.

3.2.5. Generation and Genotyping of β -Cell Specific *Sucnr1* Knockout Mice

3.2.5.1. Generation β -cell Specific *Sucnr1* Knockout Mice

To understand the specific *in vivo* role of *Sucnr1* in β -cells, we used the Cre-loxP system, as it is a powerful and widely used tool in mouse genetics. It is a site-specific recombination system derived from bacteriophage P1, and it has been engineered to allow for the manipulation of gene sequences within a living organism. The system consists of the Cre recombinase enzyme and the loxP recognition sites. The term ‘floxed’ (symbolized as fl) refers to a gene that has been mutated to be flanked by loxP sites, which are short DNA sequences that serve as recognition sites for the Cre recombinase enzyme. By integrating this system with a tissue or cell-type specific promoter, it becomes possible to achieve spatially targeted genetic manipulations⁴³⁵. In our investigation, we harnessed this by employing a tailored Cre expression system that was precisely targeted towards β -cells, thus avoiding potential confounding effects from gene deletion in other tissues.

To generate β -cell specific *Sucnr1* knockout (KO) mice (*Sucnr1*^{fl/fl} *Ins1*^{Cre/+}, also referred to as *Sucnr1*- β KO), we employed a careful and deliberate crossing strategy involving parental homozygous *Sucnr1* floxed mice (*Sucnr1*^{fl/fl} *Ins1*^{+/+}, hereafter termed as control) and *Ins1*^{Cre/+} mice⁴³⁶, which were graciously provided by Dr. Ferrer (Centre for Genomic Regulation; Barcelona, Spain). The *Ins1*^{Cre/+} mouse line was generated via a knock-in strategy that introduced the Cre recombinase gene into one of the endogenous *Ins1* alleles. Both *Ins1* and *Ins2* are expressed within mouse β -cells, yet the integration of the Cre recombinase gene into an *Ins1* allele does not significantly influence insulin secretion or the systemic glucose homeostasis within *Ins1*^{Cre/+} mice. This approach ensured a high specificity for β -cell Cre expression and, importantly, circumventing undesirable recombination events in other tissues, including the brain –a pitfall often associated with other developed β -cell-specific Cre expression systems established in prior research⁴³⁶.

The employed crossing scheme (Figure 12) started with breeding *Sucnr1* floxed mice (obtained from a previous study³⁹⁵) with the *Ins1*^{Cre/+} mice. Notably, all mice involved in this process were on a pure C57BL/6 genetic background, ensuring consistency within the study and across generations. Eventually, this strategy provided a simultaneous generation of control and *Sucnr1*- β KO mice in the same litter, which allowed for the isolation of any influence related to the *Sucnr1* gene function from potential litter effects.

3.2.5.2. Transgenic Mouse Genotyping

Genotyping is a necessary procedure in transgenic mouse studies, utilized for identifying specific alleles and validating successful recombination events. In our research, the genotyping process relied on the polymerase chain reaction (PCR) to amplify distinct genomic regions, differentiating between the floxed *Sucnr1* gene, the Cre recombinase under the *Ins1* promoter, and their wild-type counterparts. The resultant PCR products, unique in size depending on the present alleles, helped to categorize each mouse unambiguously.

Here, we used a genotyping protocol based on mouse blood lysis and DreamTaq Green DNA Polymerase kit (K1081; Thermo Fisher Scientific).

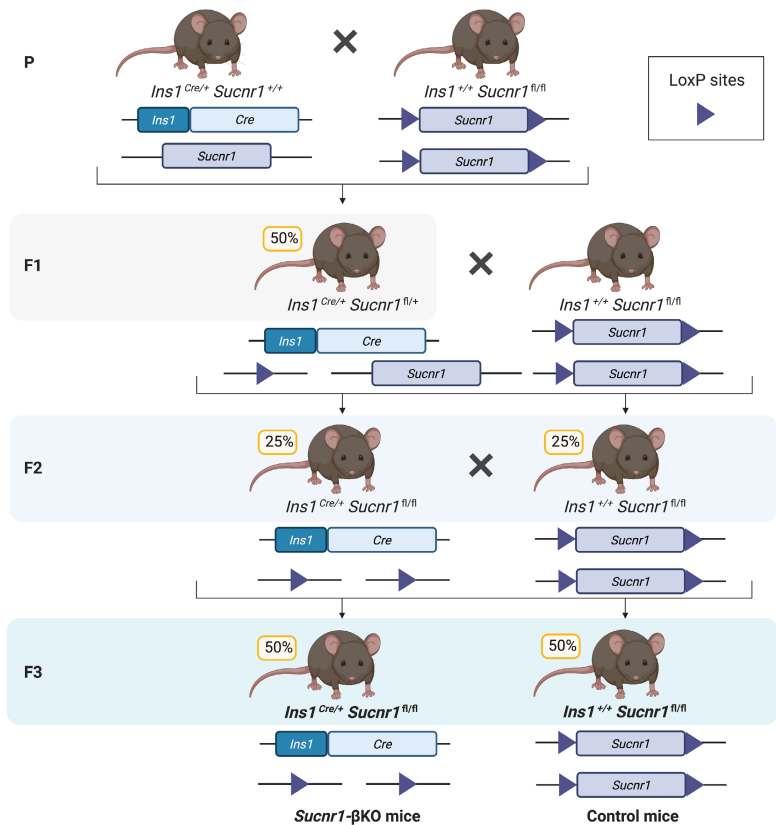


Figure 12. Breeding Strategy for the Generation of *Sucnr1*-βKO and Control Mice. This figure illustrates the multigenerational breeding plan used to produce both *Sucnr1*-βKO and control mice. It features the offspring probabilities at each stage, concluding with the F3 generation, where both experimental groups originate from the same litter.

Mouse Genotyping Protocol

1. Add 10 µl of lysis solution for blood (L3289; Sigma-Aldrich) into a 1.5 ml microcentrifuge tube.
2. Collect 5 µl of blood from tail bleeding and mix it with the lysis solution. The mixture should become darker in 2-3 minutes.
3. After 5 minutes, add 90 µl of neutralization solution for blood (N9784; Sigma-Aldrich) and mix. Store these samples at 4 °C.
4. Prepare a 20 µl PCR reaction mixture for each sample and gene. This mixture should contain 19 µl of PCR master mix prepared from the kit's components (Dream Taq Buffer and Taq DNA polymerase), deoxyribonucleotide triphosphates (dNTPs), each forward and reverse primers

for the *Sucnr1*⁺ and *Sucnr1*^{fl} alleles, and the *Ins1*⁺ or *Ins1*^{Cre} alleles, 1 µl of genotyping sample (DNA template), and nuclease-free water (Table 3). Primers' sequences are listed in Annex Table 5: Oligonucleotides, page 189). Carry out PCR reactions in a thermal cycler. Start with initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. Perform a final extension at 72 °C for 5 minutes.

Table 3. PCR Mastermix Preparation for Genotyping

Reagent	Final concentration	Volume per sample
10X DreamTaq green buffer	1X	2 µl
dNTPs 2mM	0.2 mM	2 µl
Forward primer	0.3 µM	0.6µl
Reverse primer	0.3 µM	0.6 µl
Taq DNA polymerase	62.5 mU·µl ⁻¹	0.25 µl
Nuclease-free H ₂ O	-	13.55 µl
DNA template (sample)	-	1 µl
Total	-	20 µl

5. Prepare a 1.5% agarose gel by dissolving 1.5 g of agarose in 100 ml of 1X Tris-acetate-EDTA (TAE) buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA). During gel preparation, add 6 µl of SYBR Safe DNA-binding dye (S33102; Thermo Fisher Scientific) per 100 ml of gel solution.
6. Load 10 µL of the PCR product and 7 µl of 50 ng·µl⁻¹ 100 bp DNA ladder (15628050; Thermo Fisher Scientific) into the agarose gel wells. Then, run the gel at 100V for about 30-45 minutes.
7. Visualize the gel under UV light using an iBright CL1000 Imaging System (Thermo Fisher Scientific) or a similar gel documentation equipment. The specific band sizes corresponding to different alleles allow for the identification and selection of animals carrying the desired *Sucnr1* (300 bp product for the wild-type allele and 450 bp product for the floxed form) and *Ins1* alleles (524 bp for the WT and 865 bp for the mutated structure).
8. Genotyping results should be meticulously recorded to efficiently manage and track transgenic colonies, aiding in future breeding or experimental procedures.

3.2.6. Physiological Experimentation on Mice

Physiological experimentation on animal models is a cornerstone of preclinical research, providing valuable insights into pathophysiological mechanisms. This section focuses on the methodological framework for the execution of physiological experiments, specifically glucose (GTT) and insulin tolerance (ITT) tests, on *Sucnr1*- β KO and *Sucnr1*^{fl/fl} mice.

During both tests, blood glucose was measured at the indicated times by tail bleeding using a glucose meter (Accu-Chek; Roche). Tail bleeding was the chosen blood collection method as it is a minimally invasive procedure and allows for repeated measures in conscious animals. The AUC for glucose was calculated using the trapezoidal rule for any tolerance test⁴³⁷.

3.2.6.1. Glucose Tolerance Tests

For this study, both intraperitoneal (i.p.) GTT and oral (OGTT) GTTs were conducted on the mice. For both variants, mice were fasted for 16 hours before the test. Subsequent to the fasting period, blood glucose levels were measured as a baseline reading. Glucose (2 g·kg⁻¹ body weight) was then administered either by oral gavage or *via* an i.p. injection, and blood glucose levels were measured at 15, 30, 60, and 120 minutes post-administration.

During these tests, plasma samples were collected at the baseline and 30 minutes after glucose administration. In these samples, insulin was determined using a Mouse Insulin Ultrasensitive ELISA kit (Mercodia, Uppsala, Sweden), and GLP-1 levels were analyzed in plasma using a Mouse GLP-1 ELISA kit (Crystal Chem, Elk Grove Village, IL). Both assays were performed according to the manufacturer's instructions to ensure the accuracy and reliability of the results.

3.2.6.2. Insulin Tolerance Test

The ITT is a central procedure in assessing insulin sensitivity and thereby provides crucial insight into the pathophysiology of metabolic disorders such as T2D. The ITT elucidates the efficacy of insulin-mediated glucose uptake by peripheral tissues by investigating the response to exogenous insulin administration.

The test began following a relatively short fasting period of 4 hours. This length of fasting is selected to reduce the interference from endogenous insulin or glucose re-

leased in response to recent feeding while ensuring the mice are not overly stressed or metabolically depleted, which could lead to harmful hypoglycemia during the test. After the fasting period, baseline blood glucose levels were determined using a glucose meter.

Human recombinant insulin (Actrapid, Novo Nordisk, Denmark), diluted in DPBS to ensure isotonicity, was freshly prepared immediately before the commencement of the ITT. Mice received an i.p. insulin injection at a dosage of 0.75 IU per kg body weight, a dose determined to induce a measurable response in blood glucose levels without causing severe hypoglycemia. Similar to the GTTs, blood glucose levels were tracked at 15, 30, 60, and 120 minutes after insulin administration.

3.3. Pancreas Histology

As indicated in the study, pancreatic tissues were obtained from an autopsied adult human male and mice. Tissues were washed with phosphate-buffered saline (PBS) to remove residual blood and debris and fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4 °C. Following fixation, the samples underwent dehydration and degreasing using a graded series of alcohol and xylene solutions. Once prepared, tissue samples were embedded into paraffin wax, a process that provides support to the tissue and allows for thin sectioning. The paraffin-embedded tissues were sectioned into slices of 4 µm thickness using a microtome.

Various of the obtained sections were then stained with hematoxylin and eosin (H&E), a standard staining procedure in histopathology. Hematoxylin, a basic dye, binds to acidic components of the cell, such as nucleic acids, staining them blue. In contrast, eosin, an acidic dye, stains basic elements such as cytoplasm and extracellular matrix pink. This contrast facilitates the differentiation and visualization of various cellular structures, thereby enabling a detailed examination of the tissue's histological characteristics⁴³⁸.

3.3.1. Pancreas Immunohistochemical Staining

For immunohistochemistry (IHC), formalin-fixed paraffin blocks of pancreatic tissue were sectioned at a thickness of 4 µm, and each slide was deparaffinized in xylene for 20 min, rehydrated with a decreasing ethanol series and washed with PBS. Sections were heated at 96 °C for 20 min and then incubated for 30 min with a primary antibody against SUCNR1 (1:2 dilution, NLS3476; Novus Biologicals, Centenni-

al, CO)⁴³⁹ and chromogranin A (Clone DAK-A3; Agilent, Santa Clara, CA). Automatic immunodetection was performed using the EnVision FLEX method (Agilent) with 3,3'-diaminobenzidine chromogen as substrate and hematoxylin counterstain. Panoramic 250 Flash III DX Scanner and SlideViewer 2.6 software (3DHISTEC, Budapest, Hungary) were used for image scanning, visualization, and image acquisition of stained pancreatic tissues⁴⁴⁰.

3.3.2. Pancreas Morphometric Analysis by Immunofluorescence

For morphometric analysis, at least 3 non-consecutive (150- μ m apart) 3- μ m-thick sections were obtained for each pancreas region (head, body, and tail) and analyzed per mouse. Sections were subjected to immunostaining. Briefly, slides were heated, in 10 mM sodium citrate, followed by blocking with donkey serum and incubation with primary antibodies detecting insulin (sc-8033, Santa Cruz Biotechnology, Dallas, TX) and glucagon (SAB4501137; Sigma-Aldrich). Specific signals were detected with fluorochrome-conjugated secondary antibodies with Alexa Fluor 488 (A11029) or Alexa Fluor 568 (A21043) from Thermo Fisher Scientific. Slides were counterstained with DAPI (Sigma-Aldrich) to visualize nuclei. Images were captured using a Zeiss Axio Vert A1 fluorescence microscope system (Zeiss, Oberkochen, Germany). β -cell mass was calculated by generating the ratio of the cross-sectional area of the total number of pixels from insulin-positive cells to the cross-sectional area of the total number of pixels of pancreatic tissue revealed by H&E staining, multiplied by the pancreas weight of the mouse. α -cell mass was calculated using a similar approach but using the total number of pixels from glucagon-positive cells. Images were processed and analyzed using Fiji 2.9.0 software.

3.4. *In silico* Analysis of SUCNR1 Regulation in Human Islets

The Islet Regulome Browser (IRB), an integrative data mining tool (<http://www.isle-tregulome.org>), was used in the present study to assess potential regulatory factors and elements of the *SUCNR1* gene in human pancreatic islets. The IRB is a computational resource that aggregates data from a wide range of high-throughput genomic and epigenomic studies to elucidate the potential regulatory architecture of genes in human islets. Accordingly, the datasets integrated within the IRB include whole-genome sequencing data, transcriptomic data from RNA sequencing (RNA-seq) studies, and chromatin immunoprecipitation followed by sequencing (ChIP-

seq) data for various histone modifications indicative of active regulatory elements. Additionally, the IRB contains datasets from Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), which provides information about chromatin accessibility and potential transcription factor binding sites⁴⁴¹.

The investigation of *SUCNR1* regulation within human islets using the IRB was carried out systematically. First, the *SUCNR1* locus was queried in the browser to pull up the available data across various datasets specific to this gene within the context of islet cells. The transcriptomic data provided an overview of the expression detection of *SUCNR1* in islets by RNA-seq, whereas the ChIP-seq data gave insights into the presence of histone modifications, suggesting active or repressive regulatory regions around the gene. ATAC-Seq data was analyzed to pinpoint accessible regions of chromatin in the vicinity of the *SUCNR1* gene, which could indicate potential sites for transcription factor binding.

3.5. β -Cell *In Vitro* Experimentation

3.5.1. β -Cell Lines Culture

3.5.1.1. Mouse MIN6 Cells

MIN6 is an insulinoma-derived cell line from transgenic male mice transfected with SV40 large T-antigen (SV40LT), with the ability to secrete insulin in the presence of glucose⁴⁴². MIN6 cells were maintained under standard culture conditions. Briefly, MIN6 cells were cultured at 37 °C and 5% CO₂ in DMEM consisting of 4.5 g·l⁻¹ D-glucose, 2 mM L-glutamine (Thermo Fisher Scientific), 15% fetal bovine serum (Thermo Fisher Scientific), 1% penicillin-streptomycin, 20 mM HEPES (Thermo Fisher Scientific), and 50 μ M 2-mercaptoethanol. To assess *Sucnr1* gene expression levels, cells were starved for 16 h in 2.8 mM glucose DMEM-based media, followed by stimulation with 5.5, 11.1, or 16.7 mM glucose for 3 and 24 hours.

3.5.1.2. Human EndoC- β H1 Cells

EndoC- β H1 is an engineered cell line obtained from a female human pancreas subjected to lentiviral transfection of SV40LT expressed under the control of the insulin promoter and the human telomerase reverse transcriptase (hTERT)⁴⁴³. These cells present several similarities to primary human β -cells⁴⁴⁴. EndoC- β H1 cells were

cultured as described⁴²⁹. Briefly, before seeding the cells, culture dishes and plates were coated with a mixture of DMEM (4.5 g·l⁻¹ glucose; Thermo Fisher Scientific), fibronectin (2 µg·ml⁻¹; Thermo Fisher Scientific), and extracellular matrix (1%; Sigma-Aldrich). The coated flasks were then incubated for at least 1 hour in a 5% CO₂ atmosphere at 37 °C. EndoC-βH1 cells were maintained at 37 °C and 5% CO₂ in DMEM-based media consisting of 1 g·l⁻¹ glucose, 2% BSA, 50 µM 2-mercaptoethanol (Sigma-Aldrich), 10 mM nicotinamide (Sigma-Aldrich), 5.5 µg·ml⁻¹ transferrin (Sigma-Aldrich), 6.7 ng·ml⁻¹ sodium selenite (Sigma-Aldrich), and 1% penicillin-streptomycin (Thermo Fisher Scientific). To measure SUCNR1 protein levels, cells were starved for 16 h in 2.8 mM glucose DMEM-based media, followed by treatment with 5.5, 11.1, 16.7, or 25 mM glucose for 24 h.

3.5.1.3. Primary-Like Human EndoC-βH5 Cells

The latest iteration of the human EndoC cell series is the EndoC-βH5 cell line, purchased from Human Cell Design (Toulouse, France). According to the supplier, these cells are derived from a female fetus and demonstrate an improved insulin secretion response, heightened sensitivity to incretins, and more pronounced expression of β-cell identity markers in comparison with other human β-cell lines. The generation process of these cells was similar to previous models, but the SV40LT and hTERT transgenes were removed through Cre-mediated recombination to produce mature, non-proliferative cells. EndoC-βH5 cells were seeded at 1·10⁵ cells per well in βCOAT matrix (Human Cell Design) pre-treated 96-well Cell⁺ plates (Sarstedt, Nümbrecht, Germany) and cultured for 10 days before performing the GSIS assays. Cells were maintained in ULTI-β1 medium (Human Cell Design), and the medium was changed 4 hours after seeding, when all viable cells had attached, and then again 4 days later.

3.5.2 Succinate Secretion Assay and Quantification

MIN6 cells were washed twice in Krebs-Ringer-Phosphate-HEPES buffer (KRPH; 5 mM Na₂HPO₄, 1 mM MgSO₄, 1 mM CaCl₂, 136 mM NaCl, 4.7 mM KCl, 20 mM HEPES pH 7.4 and 0.5% BSA), and then incubated for 1 h in KRPH supplemented with 2.8 mM glucose. The buffer was then replaced with KRPH containing 2.8 or 16.7 mM glucose for 1 h. The conditioned media (CM) was collected at the end of the assay was centrifuged at 500 RCF for 15 min at 4 °C, and the supernatant was used to quantify succinate content using the fluorometric EnzyChrom Succinate Assay Kit (BioAssay Systems) following the manufacturer's instructions.

3.5.3 Glucose-Stimulated Insulin Secretion Assay

In the context of the current study, GSIS assays were performed on both MIN6 mouse beta cells and EndoC- β H5 human beta cells. This allowed for a comparative understanding of the insulin secretion profiles of these cells in response to glucose concentrations and other potential stimulants or inhibitors (Figure 13). The experimental design of these assays, including the specific reagents and conditions employed, are detailed herein.

MIN6 cells were washed twice in KRPH, incubated in KRPH with 2.8 mM D-glucose for 1 h at 37°C, 5% CO₂, and saturated humidity, and then washed twice with KRPH. Cells were then incubated for 1 h in KRPH containing either 2.8 or 16.7 mM D-glucose and the corresponding stimulus.

EndoC- β H5 cells were subjected to GSIS assays following the manufacturer’s protocol (Human Cell Design). In brief, cells were starved in ULTI-ST medium for 24 hours, then washed twice in β Krebs medium (both from Human Cell Design) supplemented with 0.1% BSA (β Krebs-BSA) and incubated in β Krebs-BSA medium for 1 h at 37 °C, 5% CO₂ and saturated humidity. Cells were then stimulated for 1 h with 0 or 20 mM glucose and the indicated stimulus in β Krebs-BSA media.

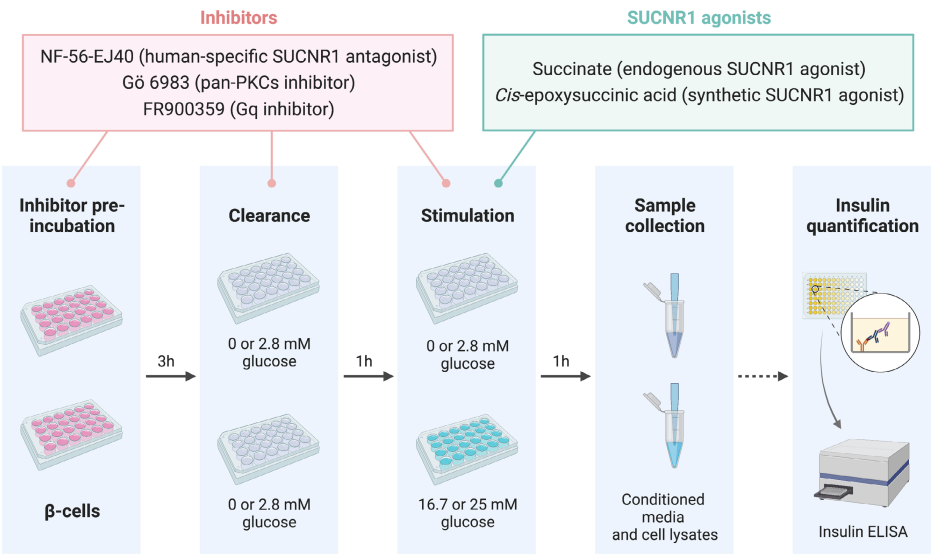


Figure 13. *In vitro* Glucose-stimulated Insulin Secretion (GSIS) Assay. The figure outlines the experimental setup for assessing GSIS, detailing the stages of the assay, employed glucose concentrations, and the application of succinate receptor 1 (SUCNR1) signaling inhibitors or SUCNR1 agonists. Insulin determination is performed with the collected samples at the end of GSIS via enzyme-linked immunosorbent assay (ELISA).

Reagents for GSIS assays were 0.5-1 mM disodium succinate (W327700; Sigma-Aldrich), 50 μ M *cis*-epoxysuccinic acid (cESA; E0449; TCI Chemicals, Tokyo, Japan), 1 μ M NF-56-EJ40 (HY-130246; MedChemExpress, Monmouth Junction, NJ), 1 μ M Gö 6983 (#2285, Tocris, Bristol, UK), and 1 μ M FR900359 (#33666, Cayman Chemical Co., Ann Arbor, MI). Inhibitors were pre-incubated for 3 h in all media preparations during the assays at the reported concentrations. At the end of GSIS assays, CM was collected and centrifuged at 300 RCF for 15 min at 4 °C, and the supernatant was stored at -20 °C. The remaining cells were washed once in DPBS and lysed with M-PER buffer with Halt Protease Cocktail (Thermo Fisher Scientific) for protein quantification. The insulin content in the CM was measured using Mouse or Human Insulin ELISA kits (Merckodia) following the manufacturer's instructions.

3.5.4. Measurement of Cytosolic Calcium

We performed detailed measurements of intracellular Ca^{2+} concentrations in MIN6 cells grown on glass coverslips (Menzel-Gläser, Germany) using the calcium-sensitive dye Fura-2 acetoxymethyl ester (Fura-2 AM).

At 80% confluency, cells were carefully removed from their growth surface by transferring the coverslip onto which they were grown to a Petri dish containing Locke-HEPES buffer (LH), composed of 120 mM NaCl, 10 mM KCl, 15 mM NaHCO_3 , 3.3 mM MgCl_2 , 2.6 mM CaCl_2 , 2.8 mM D-glucose, and 10 mM HEPES, and adjusted to a pH of 7.4. Then, cells were treated with 6 μ M Fura-2 AM (Thermo Fisher Scientific). Following incubation at 37 °C for 30 minutes, cells were thoroughly rinsed in LH buffer to remove any unincorporated Fura-2 AM.

For fluorescence measurements, coverslips with Fura-2 AM-loaded cells were carefully rinsed in LH, mounted in a specific holder (coverslip accessory L2250008; PerkinElmer, Waltham, MA), and placed in a quartz cuvette containing 1.3 ml of LH buffer. The entire setup was then placed in an LS50B PerkinElmer fluorescence spectrometer equipped with a fast-filter accessory for Fura-2 AM fluorescence ratio measurements. This equipment enabled us to monitor changes in the fluorescence emission of Fura-2 AM at 510 nm upon alternate excitation at 340 and 380 nm wavelengths, which correspond to the calcium-bound and calcium-free forms of Fura-2 AM, respectively. Thus, the ratio of the fluorescence intensities (F_{340}/F_{380}) provides a quantitative measure of the free cytosolic calcium levels in cells.

The observed changes in the F_{340}/F_{380} ratio upon extracellular succinate and glucose stimulation were recorded in real-time using proprietary software (FL WinLab 2.0;

PerkinElmer). Alterations in intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) prompted by sequential and additive stimulation with 400 μM extracellular succinate were systematically analyzed in both pre- and post-augmentation of glucose concentration to 16.7 mM. An increase over basal Ca^{2+} mobilization was assessed by AUC calculation of F_{340}/F_{380} in direct relation to the introduction of succinate, taking into account the basal glucose condition present at the moment of the respective succinate stimulus administration. This assessment allowed us to delineate the impact of the succinate stimulus on calcium mobilization above the basal level under specific glucose conditions.

3.6. Gene Expression Analysis

3.6.1. Total RNA Extraction and Quantification

3.6.1.1. RNA Extraction

RNA was extracted from tissues or cells using TRIzol Reagent (Thermo Fisher Scientific), a monophasic solution of phenol and guanidine isothiocyanate, which is able to maintain RNA integrity due to its ribonuclease (RNase) inhibitory capacity while completely lysing cells. The following protocol outlines the systematic approach to total RNA extraction used in the present study.

RNA Extraction Protocol

1. Begin by homogenizing 50–100 mg of a tissue sample, $5 \cdot 10^6$ cells, or 100–200 IEQs per 1 ml of TRIzol Reagent (Thermo Fisher Scientific). Ensure that the sample is fully immersed in the reagent for optimal results. Tissue samples can be homogenized with a tissue homogenizer. In contrast, cells and islets can be lysed directly in the culture dish or microcentrifuge tubes by adding TRIzol Reagent and passing the lysate several times through a pipette.
2. Incubate the homogenate for 5 minutes at room temperature (RT) to permit complete dissociation of the nucleoprotein complex.
3. Add 0.2 ml of chloroform (Sigma-Aldrich) per 1 ml of TRIzol Reagent. Cap the sample tubes securely and shake vigorously in a vortex for 15 seconds. Then, incubate the mixture at RT for 2–3 minutes.

4. Centrifuge the homogenate at 12,000 RCF for 15 minutes at 4 °C. The centrifugation will result in a separation into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.
5. Transfer the aqueous phase to a fresh tube with a pipette, being cautious not to disturb the interphase. Add 0.5 ml of isopropanol (Sigma-Aldrich) per 1 ml of TRIzol Reagent to the aqueous phase to precipitate the RNA. Incubate at RT for 10 minutes.
6. Centrifuge the sample at 12,000 RCF for 10 minutes at 4 °C. RNA will precipitate and form a gel-like pellet on the bottom of the tube.
7. Decant the supernatant carefully without disturbing the RNA pellet. Add 1 ml of 75% ethanol (Sigma-Aldrich), prepared using RNase-free water (Thermo Fisher Scientific), per 1 ml of TRIzol Reagent used. Vortex the sample briefly and then centrifuge at 7,500 RCF for 5 minutes at 4 °C.
8. Remove the ethanol supernatant, ensuring the RNA pellet remains undisturbed. Air-dry the RNA pellet for 5-10 minutes. Avoid over-drying the pellet, as it can significantly reduce its solubility.
9. Resuspend the RNA pellet in 20-50 µL of RNase-free water, adjusting the volume depending on the RNA amount and desired concentration. Proceed to RNA quantification or store the purified RNA at -80 °C for long-term use.

3.6.1.2. RNA Quantification

We assessed the concentration and purity of isolated RNA using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The absorbance at 260 nm provided RNA concentration. The purity of RNA was assessed by the A_{260}/A_{280} ratio, which effectively gauges the presence of proteins in the sample; aromatic amino acids present in proteins absorb light at 280 nm. Ideal RNA purity was reflected by an A_{260}/A_{280} ratio ranging from 1.8 to 2.0. Additionally, the A_{260}/A_{230} ratio can also be calculated to assess potential contaminants, such as remaining guanidine isothiocyanate. A value greater than 2.0 typically indicates relatively pure RNA.

3.6.2. cDNA Synthesis and Quantitative PCR

Complementary DNA (cDNA) was synthesized using the High-capacity cDNA Reverse Transcription kit (both from Thermo Fisher Scientific). Quantitative real-time PCR amplification (qPCR) was performed on a 7900HT Fast Real-Time PCR System with the TaqMan Gene Expression Assay from Thermo Fisher Scientific (probes are listed in **Annex Table 5: Oligonucleotides**, page 189). Relative mRNA levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method, corrected for the corresponding housekeeping gene expression levels.

cDNA Synthesis Protocol

1. Following RNA extraction, synthesize cDNA using the High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). According to the kit instructions, prepare a master mix for reverse transcription, including 2 μ l of 10X reverse transcription buffer, 0.8 μ l of 25X dNTP mix (100mM), 2 μ l of 10X reverse transcription random primers, 1 μ l of reverse transcriptase, and nuclease-free water to bring the volume up to 20 μ l per reaction.
2. Add up to 1 μ g of your RNA sample to the master mix. Mix gently and briefly centrifuge to bring the contents to the bottom of the tube.
3. Incubate the reaction mixture in a thermocycler with the following temperature profile: 25 °C for 10 minutes, 37 °C for 120 minutes, and then 85 °C for 5 minutes to inactivate the reverse transcriptase.
4. Store the resultant cDNA at -20 °C or proceed directly to qPCR.

qPCR Protocol

1. Prepare a qPCR mix containing 10 μ l of TaqMan Universal PCR Master Mix, 1 μ l of the TaqMan Gene Expression Assay probe for your gene of interest and housekeeping gene, 2 μ l of template containing 50 nmol cDNA, and nuclease-free water up to a final volume of 20 μ l.
2. Place the reaction mixture in the 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). Program the thermal cycler with the following parameters: an initial step at 50 °C for 2 minutes, a denaturation

step at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, and annealing/extension at 60 °C for 1 minute.

3. Once the qPCR is complete, the system's SDS software (Thermo Fisher Scientific) can be used to analyze the amplification plots and determine cycle threshold (Ct) values for both the gene of interest and the house-keeping gene.
4. Relative mRNA expression levels are then calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Here, ΔCt is the difference in threshold cycles for the target gene and the housekeeping gene, and $\Delta\Delta Ct$ is the difference between the ΔCt values of the test sample and a control. The formula $2^{-\Delta\Delta Ct}$ then provides the fold change in gene expression, normalized to the housekeeping gene and relative to the control. The results can be further transformed to logarithmic scale as $\log_2(\text{fold change})$ for a more straightforward interpretation and visualization of the data. In this format, positive values represent upregulation, and negative values represent downregulation of gene expression.

3.7. Protein Levels Analysis by Western Blot

3.7.1. Protein Extraction

Total protein extraction from biological samples was performed using the mammalian protein extraction reagent (M-PER) with protease and phosphatase inhibitor cocktails, both from Thermo Fisher Scientific. The extraction process utilizes M-PER, a mild detergent-based reagent, for efficient cell lysis and protein solubilization and is compatible with subsequent use in Western blot and ELISA assays.

Total Protein Extraction Protocol for Attached Cells

1. Prepare the protein lysis buffer following these proportions: 98% M-PER, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail. Keep the buffer on ice.
2. Aspirate the culture medium from the attached cells in the culture dish or wells.
3. Wash the cells twice with ice-cold PBS by adding it to the dish and gen-

tly swirling. After each wash, aspirate and discard the PBS to remove any residual culture media and detached cells.

4. Add an appropriate M-PER volume depending on the dish's size or well and the observed cell density (20-100 μ l) to the dish or wells to lyse the cells.
5. Use a cell scraper to detach the cells of the dish, ensuring all cells are collected. Transfer the cell lysate to a 1.5 mL microcentrifuge tube.
6. Incubate the tube on ice for 15 minutes to allow for complete cell lysis.
7. Following incubation, centrifuge the lysate at 14,000 relative centrifuge force (RCF) for 15 minutes at 4 °C. The high-speed centrifugation facilitates the separation of cell debris from the protein-rich supernatant.
8. Carefully collect the supernatant by transferring it to a fresh 1.5 mL microcentrifuge tube, ensuring not to disturb the cell debris pellet.
9. Protein lysates should be stored at -80 °C until further use. Multiple freeze-thaw cycles must be avoided.

Total Protein Extraction Protocol for Tissues

1. Begin by weighing the fresh or frozen tissue sample. Cut the tissue into small pieces using a clean scalpel or razor blade.
2. Add 1 mL of M-PER per 100 mg of tissue to a pre-chilled mortar. Grind the tissue thoroughly with the pestle until a homogeneous mixture is obtained. This step may take a few minutes, depending on the tissue type. Alternatively, use a tissue homogenizer.
3. Transfer the tissue homogenate to a pre-chilled 1.5 mL microcentrifuge tube.
4. Continue from step 6 for the protocol for attached cells.

Total Protein Extraction Protocol for Pancreatic Islets

1. Upon receipt of the human pancreatic islets, gently transfer them to a

15 ml conical tube using a wide-bore pipette to avoid mechanical damage.

2. Allow islets to settle by gravity for approximately 5-10 minutes at RT.
3. Carefully aspirate the supernatant, taking care not to disturb the settled islets.
4. Rinse the islets with PBS to remove any remaining culture medium. Repeat the gravity settling and supernatant aspiration process.
5. Add an appropriate volume of pre-chilled M-PER to the islets. The volume required will depend on the size and number of islets, but a general guideline is to use 100 μ L of M-PER per 1,000-2,000 IEQs.
6. Homogenize the islets gently using a pre-chilled sonicator (Bioruptor sonicator device; Diagenode Inc., Denville, NJ). Perform 10 cycles of sonication using default presets. This step will lyse the islet cells and solubilize the proteins.
7. Transfer the homogenate to a pre-chilled 1.5 ml microcentrifuge tube.
8. Continue from step 6 for the protocol for attached cells.

3.7.2. Protein Quantification

The bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific) was employed for protein quantification. First, prepare the BCA working solution according to the manufacturer's instructions.

Protein Quantification Protocol

1. Prepare 1:5 to 1:20 dilutions of the protein extract in a final volume of 10 μ L into wells of a 96-well plate. Dilutions are prepared according to the estimated protein amount.
2. Load 10 μ L of BSA protein standard from the BCA kit into dedicated wells.
3. Add 200 μ L of the BCA working solution to the tube and mix well.
4. Incubate the mixture at 37 °C for 30 minutes.

5. Allow the mixture to cool to RT.
6. Measure the absorbance at 562 nm using a spectrophotometer (Variskan LUX reader, Thermo Fisher Scientific).
7. Generate a standard curve by plotting the absorbance against the protein concentration for the standards provided with the BCA kit.
8. Use the standard curve to determine the protein concentration of your samples.

3.7.3. Western Blotting

Western blotting is a widely utilized technique in molecular biology for detecting and quantifying specific proteins in complex biological samples. This method relies on the size-based separation of proteins by gel electrophoresis, followed by their transfer onto a membrane where the target protein is detected with specific antibodies.

In the present study, the employed antibodies for Western blotting were obtained from various sources. The polyclonal primary antibody against SUCNR1 (NBP1-0086) was obtained from Novus Biologicals (Centennial, CO). The monoclonal anti- β -actin antibody was purchased from Sigma-Aldrich (A1978). Primary antibodies against phospho-PKC δ (T505) (#9374), phospho-PKC ζ/λ (T410/403) (#9378), phospho-Akt (S473) (#4058), phospho-GSK-3 α/β (S21/9) (#9331), phospho-ERK1/2 (T202/Y204) (#9101), phospho-p38 (T180/Y182) (#4511), and phospho-CREB (S133) (#9198) were obtained from Cell Signaling Technology (Danvers, MA).

The protocol for Western blotting analysis used in this thesis is detailed below.

Western Blotting Protocol

1. Begin by preparing the protein samples for gel electrophoresis. For each sample, mix 10-20 μ g of protein lysate with 5X Laemmli Sample Buffer (Bio-Rad) and 2-mercaptoethanol (Sigma-Aldrich) to a final 1X concentration. The sample buffer and β -mercaptoethanol denature the proteins and provide them with a negative charge proportional to their length.

2. Heat the samples at 95 °C for 5 minutes in a heating block to ensure complete protein denaturation. After heating, spin down the samples briefly in a microcentrifuge to collect the condensation.
3. For gel preparation, assemble the Mini-PROTEAN Tetra Cell Casting Modules (Bio-Rad) according to the manufacturer's instructions. First, pour the resolving gel solution and overlay it with isopropanol to achieve a flat gel surface. Allow it to polymerize, then remove the isopropanol and rinse the gel with 1X Tris/Glycine/SDS electrophoresis running buffer (prepared from 10X; #1610732, Bio-Rad).
4. Pour the stacking gel solution and insert the comb. The stacking gel focuses the proteins into a single band, while the resolving gel separates them according to their size.
5. Assemble the gels into the Mini-PROTEAN Electrophoresis Cell (Bio-Rad) and load the protein samples into the wells of the gel. Include a pre-stained protein ladder (PI26619, Thermo Fisher Scientific) for size reference.
6. Run the gel at 120V in electrophoresis running buffer until the dye front has reached the bottom of the gel.
7. While the gel is running, prepare the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) and chill it on ice.
8. After the electrophoresis run, disassemble the gel apparatus and carefully remove the gel.
9. Activate a polyvinylidene fluoride (PVDF) membrane (EMD Millipore) by soaking it in methanol for 1 minute. Then, rinse it with transfer buffer and keep it wet.
10. Assemble the Trans-Blot Turbo Transfer System (Bio-Rad) according to the manufacturer's instructions. Place the gel and the PVDF membrane in the transfer sandwich and immerse it in the chilled transfer buffer.
11. Run the transfer at 100V for 1.5 hours, keeping the setup cool by maintaining it on ice. This step transfers the separated proteins from the gel onto the membrane.

12. After the transfer, block the membrane with 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBS-T; 1 ml Tween 20 in 500 ml 1X TBS) for 1 hour at RT to prevent non-specific antibody binding.
13. Incubate the membrane with the primary antibody (specific to the protein of interest) diluted in the blocking solution overnight at 4 °C. The dilution factor varies depending on the antibody and previous observations, which is usually 1:1,000.
14. The next day, wash the membrane three times for 10 minutes each with TBS-T in agitation to remove unbound primary antibodies.
15. Incubate the membrane with the secondary antibody, conjugated to horseradish peroxidase (HRP), diluted 1:2,000 in TBS-T for 1 hour at RT in mild agitation.
16. Following secondary antibody incubation, wash the membrane three times for 10 minutes each with TBS-T to remove unbound secondary antibodies.
17. Prepare the chemiluminescent Pierce ECL Western Blotting Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) according to the manufacturer's instructions.
18. Apply the substrate to the membrane, ensuring even coverage. The HRP enzyme on the secondary antibody will react with the substrate and produce light.
19. Detect the chemiluminescent signal using an iBright CL1000 Imaging System (Thermo Fisher Scientific) or similar instrument. The intensity of the luminescent signal is directly proportional to the amount of target protein present on the membrane.
20. For protein quantification, analyze the intensity of the bands using iBright Analysis Software (Thermo Fisher Scientific) or similar image analysis software.
21. If required, strip the membrane using Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) and re-probe with antibodies.

3.8. Statistical Analysis

The data presented in tables and graphics are displayed as either the mean $\pm$ standard deviation (SD) or mean $\pm$ standard error mean (SEM), as indicated. The AUCs were calculated using the trapezoid rule. In the box-and-whisker plots, the median is indicated by a center line, while the box limits represent the upper and lower quartiles and the minimum to the maximum range of the data set is indicated by the whiskers.

The Shapiro-Wilk normality test was applied before any contrast hypothesis testing. When the data were normally distributed, a two-tailed Student's *t*-test was used to compare differences between two groups, applying Welch's correction if a difference between the variations of the compared groups was observed, whereas a one-way analysis of variance (ANOVA) with Dunnet's *post hoc* test was used to compare multiple groups to a control group. For time-course experiments, a two-way ANOVA with Bonferroni's correction for multiple comparisons was conducted.

In cases where the data did not follow a normal distribution, the non-parametric Mann-Whitney two-tailed *U*-test for unmatched pairs or the Wilcoxon signed-rank test for matched pairs were used to compare differences as appropriate.

Pearson's or Spearman's correlations were employed to calculate linear associations between continuous variables.

Both GraphPad Prism 9 and RStudio software for Mac OS and IBM SPSS 27 software for Windows OS were utilized for the statistical analyses, and a *p*-value of less than 0.05 was considered statistically significant.

UNIVERSITAT ROVIRA I VIRGILI

THE SUCCINATE-SUCNR1 AXIS AND ITS IMPACT ON BETA-CELL FUNCTION: AN INTEGRATIVE STUDY

UNCOVERING NOVEL INSIGHTS INTO INSULIN SECRETION REGULATION AND DIABETES PATHOGENESIS

Joan-Martí Sabadell i Basallote

Results

4

UNIVERSITAT ROVIRA I VIRGILI

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4.1. Characterization of SUCNR1 Expression in the Pancreas and Islets

The expression of SUCNR1 in the pancreas and islets has been previously reported, albeit at low levels in comparison with other tissues, such as white adipose tissue and the liver^{445–447}. Although the exact role of this receptor in the pancreas is still unexplored, it might play a pivotal part in the regulation of insulin release and β -cell function, thus warranting further investigation.

4.1.1. Examination of SUCNR1 expression in the pancreas and islet cell populations

Our initial analysis focused on the expression of *Sucnr1* mRNA across various metabolic tissues harvested from mice, which has recapitulated the pattern previously reported in the literature^{445–447}, confirming the expression of *Sucnr1* in the pancreas (Figure 14A). To enhance the resolution of our understanding of SUCNR1 distribution, we performed immunohistochemical (IHC) staining in human and mouse pancreas sections. This allowed us to discern a differential abundance of the SUCNR1 protein in the pancreatic islets compared to exocrine tissues. Notably, a qualitatively higher protein abundance was observed within islets in both human and mouse samples (Figure 14B). To further delve into the cell type-specific expression of SUCNR1 within pancreatic islets, we examined its mRNA levels in the two principal cell populations: α - and β -cells. Our data revealed that *Sucnr1* was primarily expressed in β -cells derived from rat islets (Figure 14C), suggesting a prospective role of this receptor in β -cell physiology.

4.1.2. *In silico* Assessment of SUCNR1 Expression Regulation in Human Islets

The regulatory mechanisms of gene expression can vary markedly across different tissues, and specific factors may influence the expression of *SUCNR1* in human islets. To explore this, we utilized the Islet Regulome Browser (IRB), a comprehensive and integrative tool that facilitates the study of gene regulatory mechanisms in human islets⁴⁴¹. This *in silico* analysis identified the transcription factors NK2 homeobox 2 (NKX2.2), forkhead box protein A2 (FOXA2), and NK6 homeobox 1 (NKX6.1) as potential regulators of *SUCNR1* expression. These transcription factors are recognized for their crucial role in maintaining β -cell identity and function^{448–450} (Figure 15). For instance, NKX2.2 is essential for the development of β -cells and is involved in

Results

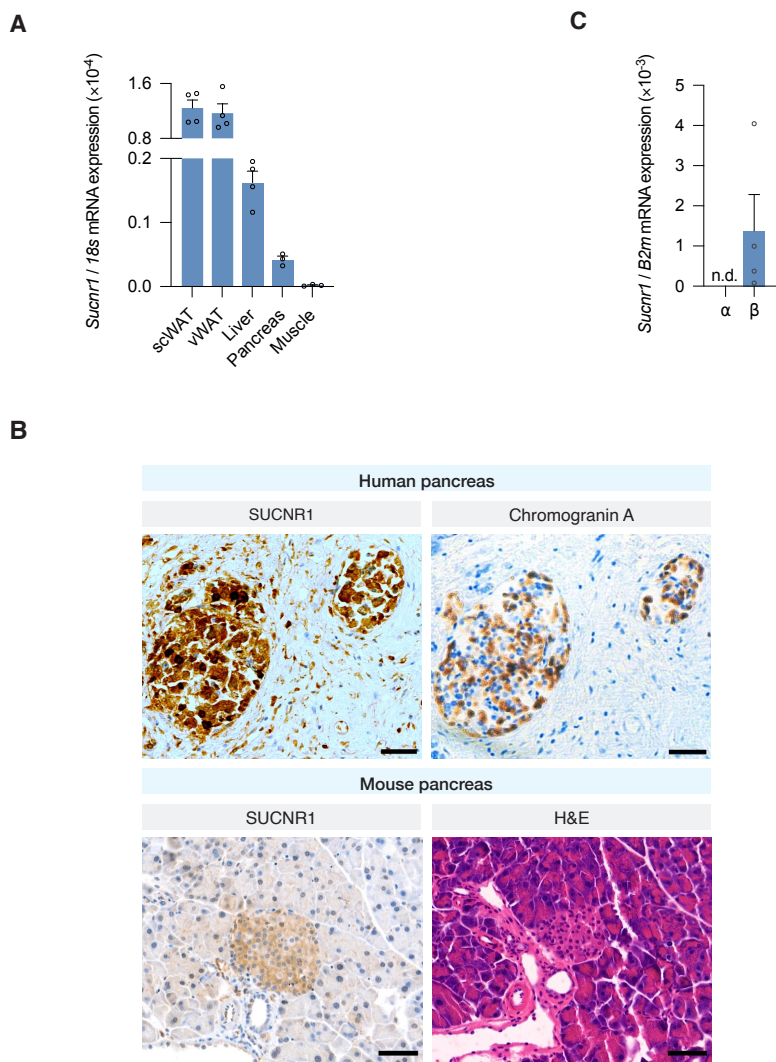


Figure 14. SUCNR1 Expression in the Pancreas and Islets. **A)** *Sucnr1* mRNA levels were analyzed in subcutaneous white adipose tissue (scWAT), visceral WAT (vWAT), liver, pancreas, and skeletal muscle by qPCR (n=3-4). **B)** Immunohistochemical (IHC) staining of SUCNR1 in human and mouse pancreas sections, and chromogranin A IHC staining or hematoxylin and eosin (H&E) staining. Scale bars represent 50 μ m. **C)** Analysis of *Sucnr1* mRNA expression in α - and β -cells isolated by fluorescence-activated cell sorting (FACS) from rat islets (n=4). Not detected (n.d.). Data are presented as mean $\pm$ SEM.

insulin gene transcription^{450,451}. FOXA2 is known to play a vital role in pancreatic development and is critical for insulin secretion, impacting the metabolic function of β -cells⁴⁵⁰. Lastly, NKX6.1 is a key factor for β -cell development and mature differentiation and is necessary for β -cell proliferation and GSIS^{450,452}. Therefore, the involvement of these transcription factors suggests a potential role of *SUCNR1* in β -cell function.

Additionally, by using the IRB, we could perform a bioinformatics analysis of a published RNA sequencing (RNA-seq) library from human islets⁴⁵³, further corroborating the detection of *SUCNR1* transcripts in this endocrine compartment. Notably, we discovered that the *SUCNR1* locus and its nearby regions contain several single-nucleotide polymorphisms (SNPs). These SNPs had been previously associated with T2D in patients, as indicated by an accessible dataset from a genome-wide association study which we examined via the IRB⁴⁵⁴ (Figure 15). However, the potential implications of these SNPs in the development of T2D and its impact on *SUCNR1* expression and function will require further exploration.

Overall, these findings laid the groundwork for further investigation into the functional role of *SUCNR1* in β -cell physiology and the potential implications for glucose homeostasis regulation.

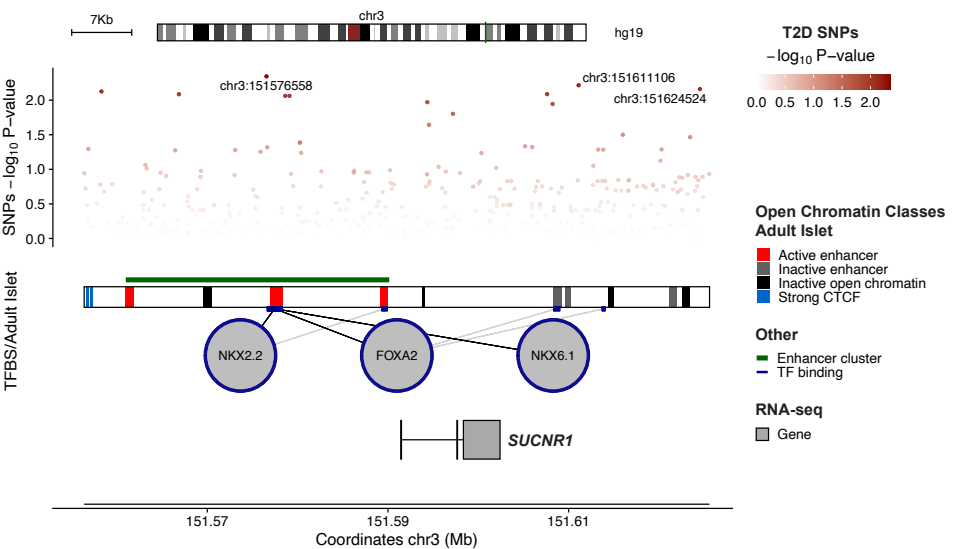


Figure 15. *In silico* Analysis of *SUCNR1* Regulation in Human Islets. *In silico* study of *SUCNR1* gene expression regulation by genomic sequences and specific human adult islet transcriptional factors, and single nucleotide polymorphisms (SNPs) associated with type 2 diabetes (T2D) localized within or surrounding the *SUCNR1* locus. CTCF: CCCTC-binding factor; NKX2.2: NK2 homeobox 2; Forkhead box protein A2; NKX6.1: NK6 homeobox 1; SNPs: Single nucleotide polymorphisms; TF: transcription factor; TFBS: transcription factor binding site.

4.2. Evaluating SUCNR1 Expression in Human Islets During Obesity and T2D

As previously detailed, the comprehensive characterization of *SUCNR1* in pancreatic islets unveils the potential of this receptor as a significant player in the machinery of β -cells. Moreover, the substantial involvement of *SUCNR1* in physiology and disease in various metabolic organs²⁷² raises the question of how *SUCNR1* levels in the islets might be affected by different metabolic conditions such as obesity and T2D.

To illuminate this query, we investigated *Sucnr1* expression in whole pancreatic tissue from rodent models of obesity and T2D. Notably, a rising trend of *Sucnr1* expression was observed in diet-induced obese (DIO) mice, suggesting an upregulated transcriptional response to increased metabolic demand. This trend was further amplified in *db/db* mice, which served as a model of T2D (Figure 16A). Briefly, *db/db* mice harbor a mutation in the leptin receptor gene (*Lepr^{db/db}*). This mutation impairs the signaling pathway of leptin, a hormone responsible for regulating appetite and energy expenditure. As a result, *db/db* mice exhibit hyperphagia and reduced energy expenditure, leading to severe obesity and features of insulin resistance and hyperglycemia, resembling the pathophysiology observed in T2D⁴³⁴. This trend observed in rodent models may hint at a potential adaptive mechanism in response to obesity and T2D, whereby increased *Sucnr1* expression might modulate insulin secretion to compensate for increased metabolic demand and insulin resistance.

To explore our findings in a human context, we took the investigation a step further by analyzing pancreatic islets from deceased donors who were either obese or diagnosed with T2D in two independent cohorts. Notably, acquiring human islet samples is challenging due to their scarcity, the involved ethical considerations, logistical hurdles in procurement, and the need for careful handling to maintain their quality. Human islet samples are hard to come by as suitable donors are few, and their availability depends on infrequent occasions where pancreas tissue can be harvested from deceased individuals. Moreover, the delicate nature of these samples demands stringent quality control measures and specialized expertise to ensure their viability during handling, reinforcing their value to researchers^{455,456}.

Consistent with our findings from rodent models, significantly higher *SUCNR1* expression was observed in human obese islets compared to their healthy counterparts, whereas no significant changes were observed in the expression levels of *GLP1R*, a major modulator of insulin secretion in β -cells under these metabolic con-

ditions (**Figure 16B**; donor information in **Table 1** in the **Materials and Methods chapter**, page 76).

To ensure that these changes in mRNA expression were translated into protein, we confirmed these results by western blot in human islet lysates obtained from an additional cohort, including donors with obesity and T2D (**Figure 16C**, donor information in **Table 2** in the **Materials and Methods chapter**, page 77). The congruity of these findings underscores the potential involvement of SUCNR1 in the pathophysiology of obesity and T2D.

Furthermore, we discovered a positive correlation between the levels of SUCNR1 mRNA and protein in islets and the BMI of the human donors (**Figure 16D**). This association suggests that an increased metabolic burden, as indicated by a higher BMI, may stimulate the upregulation of SUCNR1 expression in islets, potentially reflecting an adaptive response of β -cells to metabolic stress.

Taken together, these findings propose a compelling link between islet SUCNR1 expression and changes in metabolic status, further hinting at the prospective role of this receptor in the pathophysiology of metabolic disorders and T2D.

4.3. Effects of the Succinate-SUCNR1 Axis on Insulin Secretion Regulation

Following our observations of the exclusive SUCNR1 expression in β -cells within islets, coupled with its upregulation in human islets under metabolic conditions like obesity and T2D, curiosity arises regarding its functional importance within these cells. Since β -cell functionality is central to both glucose homeostasis and the development of T2D, acquiring insight into the role of SUCNR1 within this context becomes essential.

4.3.1. Investigation into Glucose-Mediated Modulation of Succinate Release and SUCNR1 Expression in β -Cells

Our studies were initiated by investigating how hyperglycemia could potentially link to the succinate-SUCNR1 axis in β -cells. Intracellular succinate levels rise in β -cells during glucose catabolism principally through pyruvate oxidation *via* the TCA cycle³¹⁸. In this context, our findings revealed that high-glucose conditions stimulated succinate release in the mouse insulinoma-derived β -cell line MIN6 (a

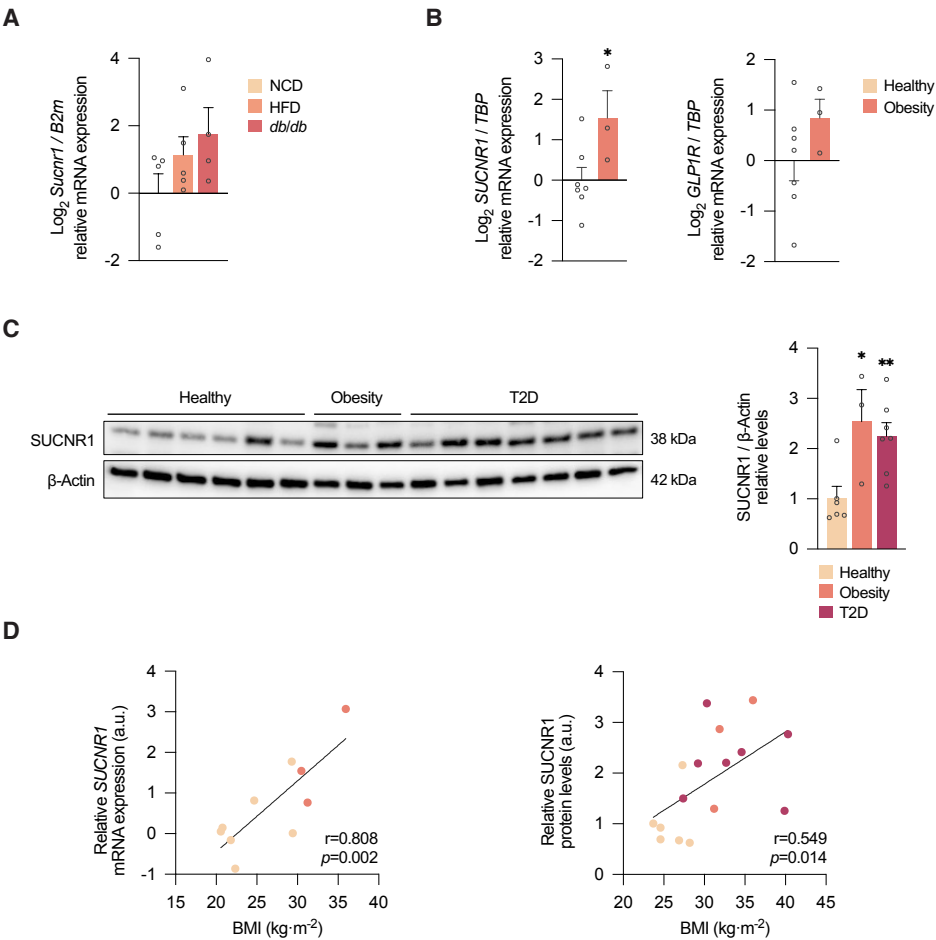


Figure 16. SUCNR1 Levels Analysis in Islets from Donors with Obesity and Type 2 Diabetes (T2D). **A)** *Sucnr1* mRNA expression in the entire pancreas of normal-chow diet (NCD)- and high-fat diet (HFD)-fed wild-type mice, and *db/db* mice on NCD (n=4-5). **B)** *SUCNR1* and *GLP1R* mRNA expression in islets from healthy individuals (n=7) and donors with obesity (n=3). **C)** *SUCNR1* protein levels in human islet lysates from healthy (n=6) and donors with obesity (n=3) and T2D (n=7). **D)** Linear correlations between the body mass index (BMI) of donors and *SUCNR1* mRNA (n=10) and protein (n=16) expression. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$ vs. control (Student's t-test in **A-C**, or Pearson's correlation coefficient in **D**). BMI: body mass index. *B2m*: β2 microglobulin; *TBP*: TATA box binding protein.

20% increase compared to low glucose; **Figure 17A**). Moreover, a dose-dependent increase in *Sucnr1* expression was observed in response to different glucose concentrations in MIN6 cells (**Figure 17B**), a result further corroborated at the protein level in the human β-cell line EndoC-βH1 (**Figure 17C**). These results imply a connection between hyperglycemia and the succinate-SUCNR1 axis in β-cells, underpinned by glucose-dependent augmentation in succinate release and SUCNR1 expression in both mouse and human β-cell lines.

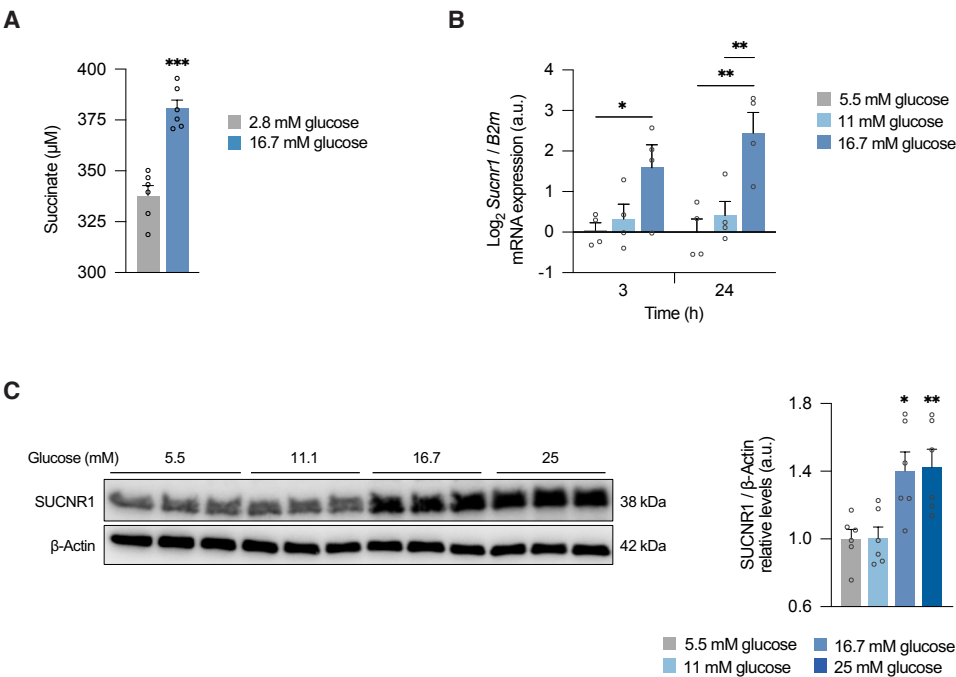


Figure 17. Glucose Regulation on Succinate Release and SUCNR1 Expression in β-Cells. **A)** Succinate quantification in the conditioned media of MIN6 cells cultured in low- or high-glucose conditions (n=4). **B)** *Sucnr1* mRNA expression in MIN6 cells stimulated with different concentrations of glucose for 3 or 24 hours (n=4). **C)** SUCNR1 protein levels in EndoC-βH1 cells stimulated with different concentrations of glucose for 24 hours (n=6). Data are presented as mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001 vs. basal conditions or indicated comparisons (Student's *t*-test in **A** or ANOVA with Dunnett's test for multiple comparisons in **B** and **C**).

4.3.2. Assessment of SUCNR1 Activation in Insulin Secretion

Having established a glucose-responsive dynamic between succinate release and SUCNR1 expression, we explored the effect of SUCNR1 activation on insulin secretion in glucose-stimulated insulin secretion (GSIS) assays. To isolate the effect of extracellular succinate, we also employed the synthetic SUCNR1 agonist *cis*-epoxy-succinic acid (cESA), which has a lower half maximal effective concentration (EC₅₀) compared to succinate and cannot be metabolized intracellularly⁴⁵⁷, thereby circumventing any potential intracellular effects of succinate induced by its uptake into cells *via* dicarboxylic acid transporters^{327,328} (**Figure 18A**). Insulin secretion was significantly enhanced by extracellular succinate under glucose-stimulated conditions in MIN6 cells. Additionally, results showed that cESA similarly augmented insulin secretion under high glucose (**Figure 18B**).

Our observations were replicated in the primary-like human β -cell line EndoC- β H5, a cell line that does not present limitations regarding the expression of certain GPCRs important for β -cell function, including GLP-1R⁴⁵⁸, and have an enhanced GSIS response compared to other *in vitro* β -cell lines^{459,460}. EndoC- β H5 cells showed an increase in insulin secretion when SUCNR1 was activated by both extracellular succinate or cESA under conditions of no glucose (1.5-fold or 1.3-fold increase, respectively) and high-glucose (1.4-fold increase for both) conditions (**Figure 18C**).

To further demonstrate that this succinate effect on insulin release depends on its extracellular-driven signaling properties, we co-treated EndoC- β H5 cells with the human-specific SUCNR1 antagonist NF-56-EJ40 3-hours before and during the GSIS assay. Briefly, NF-56-EJ40 is a potent and highly selective antagonist of the human SUCNR1 receptor. It exhibits a high affinity for human SUCNR1, with a half-maximal inhibitory concentration (IC_{50}) of 25 nM. Intriguingly, we discovered that succinate combined with acute receptor antagonization *via* NF-56-EJ40 could stimulate insulin release in EndoC- β H5 cells even in the absence of glucose, hinting at a mechanism warranting investigation. Conversely, in the presence of high glucose, succinate-induced insulin secretion was entirely inhibited by the antagonist (**Figure 18D**), reaffirming the critical role of SUCNR1 in mediating the insulintropic effects of extracellular succinate.

4.3.3. Dissecting the SUCNR1 Signaling Transduction Mechanism in β -Cells

We further delved into the mechanistic underpinnings of SUCNR1-mediated insulin secretion by focusing on the involved transduction pathways. Notably, the signaling pathways associated with SUCNR1 are characterized by their cell- and context-dependent nature, a finding well-documented in the literature^{272,461}. This inherent complexity guided our experimental approach, which sought to be comprehensive in scope.

We thus challenged MIN6 cells with succinate or cESA and analyzed several canonical proteins that might integrate GPCR signal transduction. Upon stimulating MIN6 cells with these agonists, we found a SUCNR1-dependent increase in the phosphorylated forms of Akt and novel and atypical PKCs, PKC δ and PKC ζ , respectively. The activation of these proteins coincided with differential phosphorylation of ERK1/2 and p38 MAPK. Concurrently, an increase in phosphorylated cAMP response element-binding protein (CREB) and phosphorylated (inactive) glycogen synthase ki-

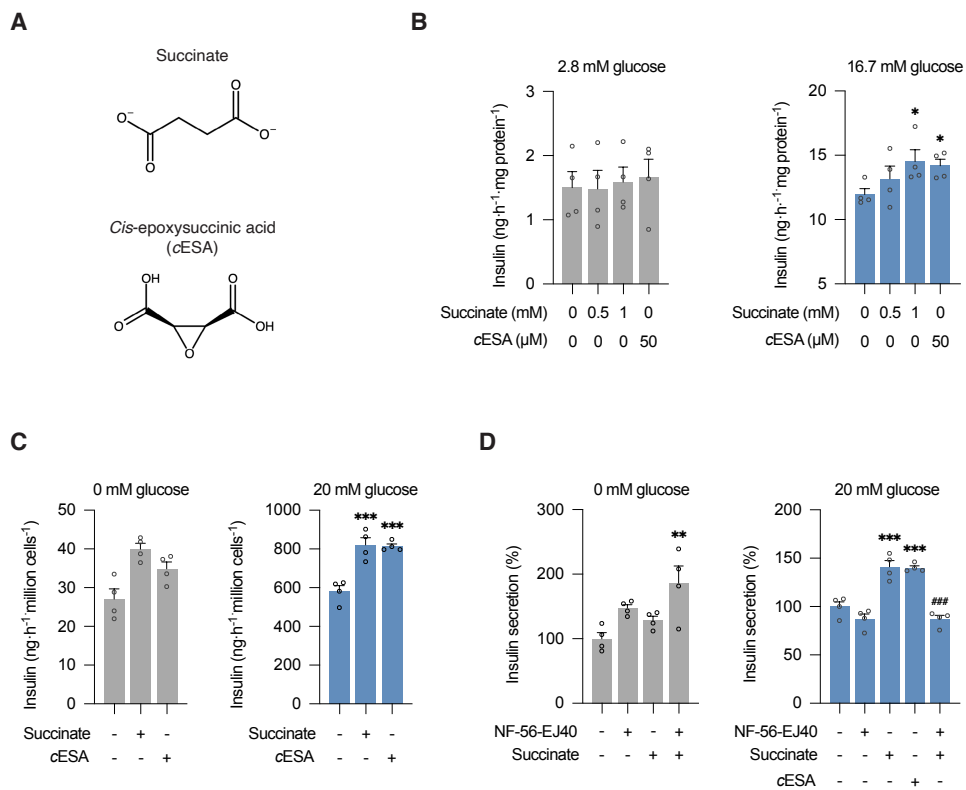


Figure 18. Impact of the Succinate-SUCNR1 on Glucose-Stimulated Insulin Secretion. A) Chemical structures of succinate and *cis*-epoxysuccinic acid (cESA). **B)** Insulin quantification in the conditioned media (CM) of MIN6 cells stimulated with succinate or cESA at 2.8 mM or 16.7 mM glucose (n=4). **C)** Insulin secretion in glucose-stimulated insulin secretion (GSIS) assays in Endo-CβH5 cells stimulated with 500 μM succinate or 50 μM cESA at 0 mM or 20 mM glucose, determined in the CM by ELISA (n=4). **D)** Insulin secretion in Endo-CβH5 cells incubated with a human-specific SUCNR1 antagonist (1 μM NF-56-EJ40) and 500 μM succinate or 50 μM cESA (n=4). Data are presented as mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001 vs. basal conditions; ###*p*<0.001 vs. succinate (ANOVA with Dunnett's test for multiple comparisons in **B-D**).

nase-3α/β (GSK-3α/β) was observed, implicating these proteins in SUCNR1-mediated signaling (**Figure 19**). This concomitant modulation in phosphorylation levels may involve these proteins as significant players in SUCNR1-mediated signaling.

Considering the known sensitivity of β-cells to fluctuations in Ca²⁺ concentrations, we conducted a comprehensive examination of Ca²⁺ mobilization subsequent to SUCNR1 activation. We utilized a fluorometric assay, a technique commonly employed for quantitatively measuring real-time changes in intracellular Ca²⁺ concentrations ([Ca²⁺]_i). In the initial phase of our experiment, we challenged MIN6 cells cultured under low-glucose conditions with increasing doses of succinate. Interestingly, this exposure elicited no observable changes in [Ca²⁺]_i, indicating that low glu-

cose conditions were not conducive to a response. We then shifted our experimental conditions, increasing the glucose concentration in the culture medium to 16.7 mM.

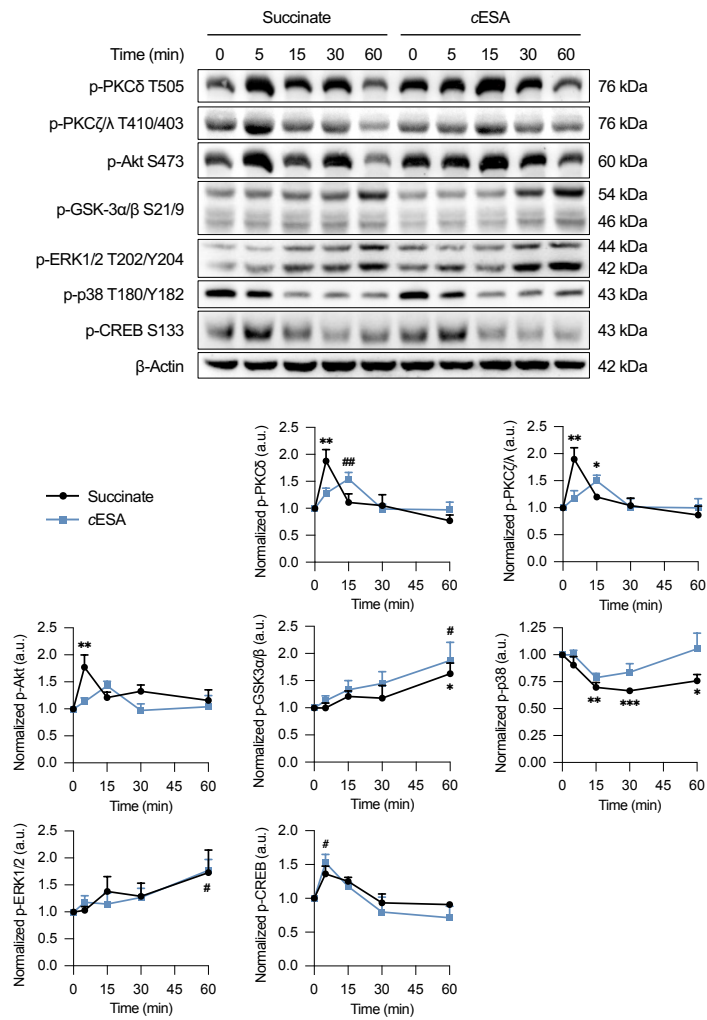


Figure 19. SUCNR1 Activation in β-cells and its Influence on Downstream Signaling. Western blot analysis of several phosphoproteins in MIN6 cells stimulated with 500 μM succinate or 50 μM *cis*-epoxysuccinic acid (cESA) at different time points (n=4-5). Data are presented as mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001 when comparing succinate with the basal condition; #*p*<0.05, ##*p*<0.01 when comparing cESA with the basal condition (ANOVA with Dunnett's test for multiple comparisons).

Notably, under these high-glucose conditions, intracellular Ca^{2+} was progressively mobilized in response to increasing doses of succinate (ANOVA after glucose addition $p=0.0126$), underscoring the role of high-glucose presence as a crucial modulator in this response (Figure 20A). As cells were exposed to increasing succinate levels before high-glucose stimulation, we studied the succinate effect on Ca^{2+} mobilization immediately after glucose addition in an additional set of experiments. We

also observed a significant glucose-dependent succinate dose-response on $[Ca^{2+}]_i$ in MIN6 cells at lower extracellular succinate doses, which reached significance at 800 μ M succinate (ANOVA after glucose addition $p=0.0013$) (Figure 20B). The dose-response relationship between extracellular succinate and $[Ca^{2+}]_i$ under high-glucose conditions points to a dynamic interplay between these factors in regulating insulin secretion in β -cells. These findings add a new dimension to our understanding of SUCNR1-mediated insulin secretion, indicating the modulation of intracellular Ca^{2+} as a key event in this process.

The observed activation of PKC isoforms during the western blotting analysis underpins their significant role in GPCR-stimulated insulin secretion within the context of neurohormonal amplification pathways^{462,463}. In line with this, we sought

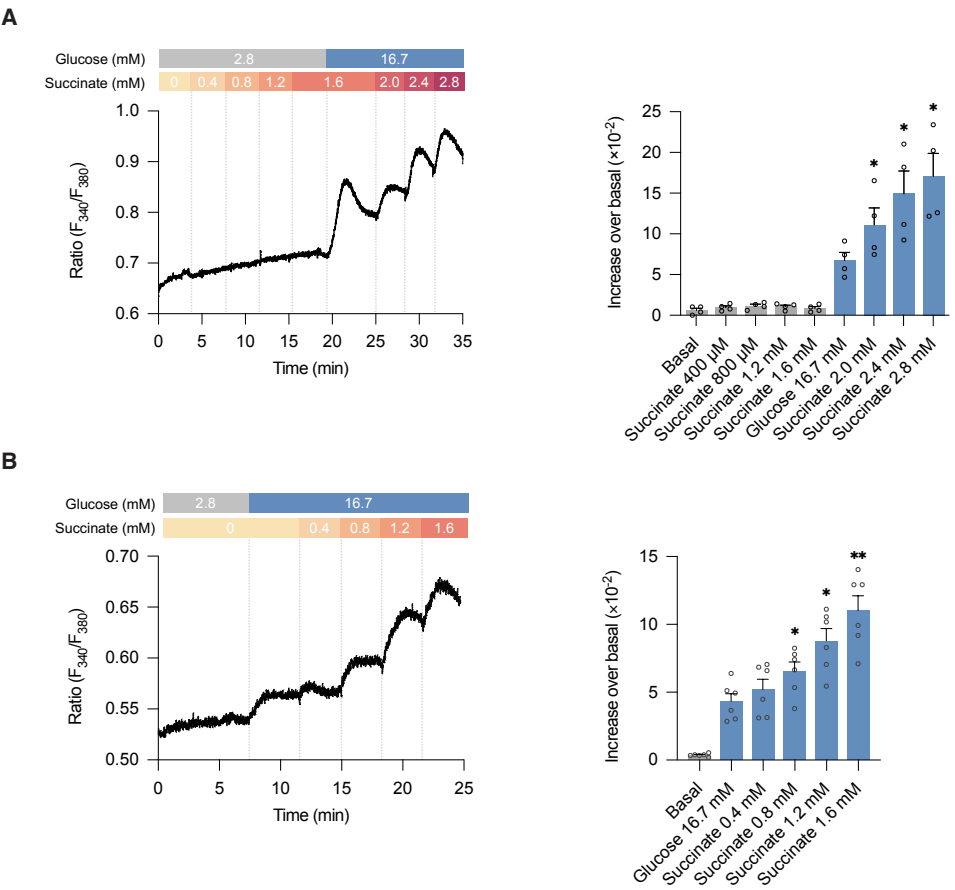


Figure 20. Effects of Extracellular Succinate on Intracellular Ca^{2+} Mobilization in β -Cells. Ca^{2+} mobilization was monitored in real-time using the Fura-2 acetoxymethyl ester fluorometric ratio in MIN6 cells stimulated with additive succinate doses. **A)** Succinate was added before and after glucose stimulation, as indicated by the doses and the interval lines on the graph ($n=4$). **B)** Succinate was added after glucose stimulation ($n=6$). Data are presented as mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$ (ANOVA with Dunnett's test for multiple comparisons).

to discern the role of this family of serine-threonine kinases in SUCNR1-induced insulin secretion. The pan-PKC inhibitor Gö 6983 was employed in a GSIS, resulting in a partial blockade of succinate-stimulated insulin release under high-glucose conditions in EndoC- β H5 cells. This strongly indicates the involvement of the PKC pathway in the SUCNR1-mediated potentiation of insulin secretion (**Figure 21A**).

This led us to probe upstream of PKC, focusing on Gq proteins, known mediators of GPCR signaling upon receptor activation⁴⁶⁴. Our data revealed a complete blockade of succinate-induced potentiation of insulin release following inhibition of Gq activity by using the chemical compound FR900359, indicating a critical role of Gq in mediating the insulinotropic effect of SUCNR1 (**Figure 21B**).

Overall, our findings collectively underscore the significance of the succinate-SUCNR1 axis in regulating insulin secretion, an effect mediated by Gq coupling and the subsequent Ca^{2+} mobilization and PKC pathways in β -cells (**Figure 21C**).

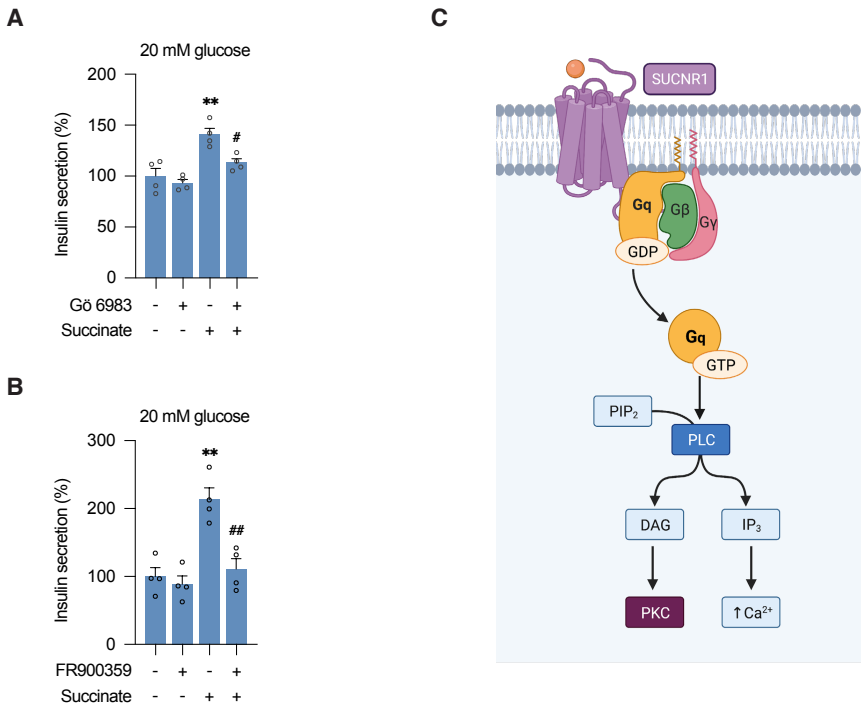


Figure 21. Analysis of Gq and PKC Pathways Influence on SUCNR1-Mediated Insulin Secretion. In high glucose conditions, EndoC- β H5 cells were treated with **A**) 1 μM of PKC inhibitor Gö 6983, followed by incubation with 500 μM succinate ($n=4$), and **B**) 1 μM FR900359 to inhibit Gq activity, followed by treatment with 500 μM succinate ($n=4$). Insulin levels were determined in the conditioned media at the end of the glucose-stimulated insulin secretion assays. **C**) Proposed mechanism of SUCNR1 signaling in β -cells. Data are presented as mean $\pm$ SEM. ** $p<0.01$ when comparing succinate with the basal condition; # $p<0.05$, ## $p<0.01$, when comparing an inhibitor with succinate (ANOVA with Tukey's test for multiple comparisons in **A** and **B**).

4.4. Unveiling the *In Vivo* Significance of SUCNR1 in β -Cells for Insulin Secretion Regulation

4.4.1. Generation of a β -Cell-Specific SUCNR1 Knockout Mouse Model

To gain a more comprehensive understanding of the role of SUCNR1 in the context of pancreatic β -cells *in vivo*, we generated a mouse model with β -cell-specific *Sucnr1* deletion. This was achieved by employing Cre/loxP-mediated recombination, which has been extensively proven to be an indispensable technology for gene manipulation with spatial precision⁴³⁵. We crossed mice carrying a conditional *Sucnr1* loxP-flanked locus (*Sucnr1*^{fl/fl})⁴¹² with heterozygous transgenic mice in which the Cre recombinase gene is inserted at the initiator codon of the endogenous *Ins1* gene locus (*Ins1*^{Cre/+}). *Ins1*^{Cre/+} mice do not exhibit recombination in the brain and maintain similar glucose homeostasis and body weight gains compared to wild-type mice, allowing us to focus on the role of SUCNR1 in β -cells specifically⁴³⁶. The offspring of these breedings included both control mice (*Ins1*^{+/+} *Sucnr1*^{fl/fl}, referred to as *Sucnr1*^{fl/fl}) and mice with β -cell-specific *Sucnr1* ablation (*Ins1*^{+/+} *Sucnr1*^{fl/fl}, referred to as *Sucnr1*- β KO mice) (Figure 22A). These mice were born following typical Mendelian inheritance patterns, with no apparent morphological differences between genotypes. Immunohistochemical staining confirmed a qualitatively significant decrease in SUCNR1 content within the pancreatic islets of *Sucnr1*- β KO mice (Figure 22B).

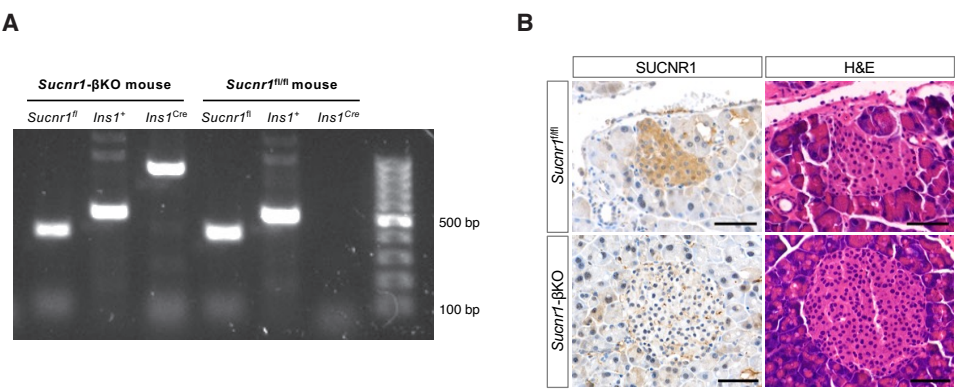


Figure 22. Generation of *Sucnr1*- β KO Mice. **A)** Genotyping PCR results displaying the amplified alleles for each mouse genotype (*Sucnr1* floxed allele 450 bp; *Ins1* wild-type allele 524 bp; *Ins1* mutated allele 865 bp; bp: base pairs). **B)** Pancreas immunohistochemical staining of SUCNR1, accompanied by serial hematoxylin and eosin staining (H&E), showing a qualitative decrease in SUCNR1 protein levels in islets of *Sucnr1*- β KO mice compared to control mice. Scale bar represents 50 μ m.

4.4.2. Examination of β -Cell-Specific SUCNR1 Deletion in Adult and Aged Mice on a Normal-Chow Diet

On a normal control diet (NCD), we did not observe any significant differences between the generated genotypes in terms of body weight, organ weight, glucose tolerance, insulin sensitivity, or insulin secretion at 20 (Figure 23) and 54 weeks of age (Figure 24). However, *Sucnr1*- β KO aged mice presented a tendency towards a worse glucose profile (Figure 24B) and glucose intolerance during an oral glucose tolerance test (Figure 24E) compared to control mice. These data indicate that in the absence of overt metabolic stress, β -cell-specific *Sucnr1* deletion did not significantly impact these metabolic parameters.

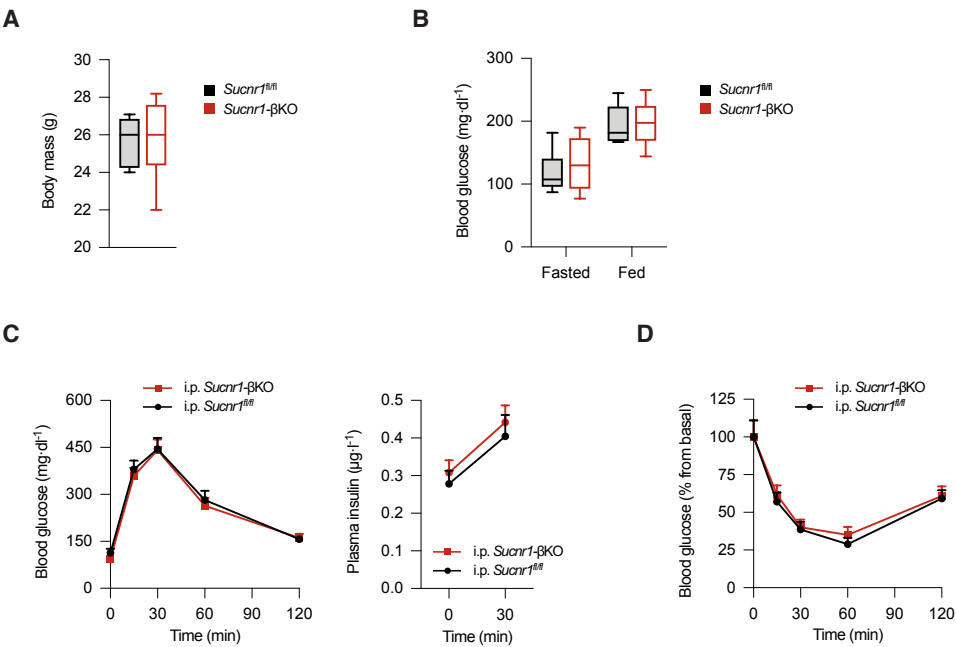


Figure 23. *Sucnr1*- β KO mice Under Normal-Chow Diet (NCD) at 20 Weeks of Age. **A)** Body mass of control and *Sucnr1*- β KO mice (n=8-10). **B)** Blood glucose levels in control and *Sucnr1*- β KO mice in fasted or random-fed conditions. **C)** Intraperitoneal glucose tolerance test in control and *Sucnr1*- β KO mice; displayed are the blood glucose (n=8-10) and plasma insulin levels (n=6-7) during the test. **D)** Insulin tolerance test in control and *Sucnr1*- β KO mice (n=8-10). Data are displayed as mean $\pm$ SEM.

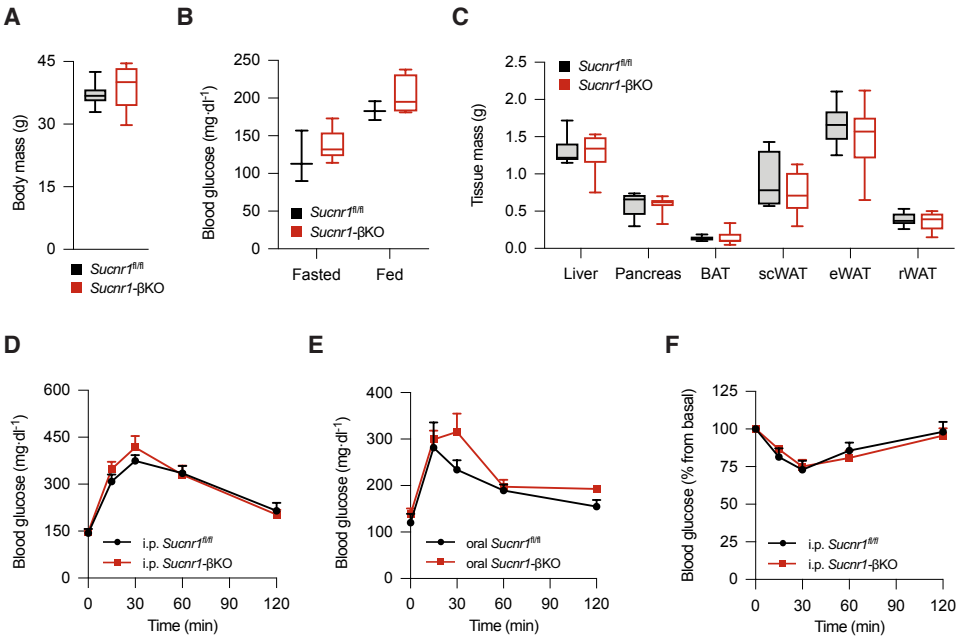


Figure 24. Old-Age *Sucnr1*-βKO Mice on Normal-Chow Diet (NCD). **A**) Body mass of control and *Sucnr1*-βKO mice under NCD at 54 weeks (n=7-8). **B**) Blood glucose levels in control and *Sucnr1*-βKO mice in fasted or random-fed conditions (n=3-4). **C**) Tissue mass at the end of the study at 54w (n=7-9). Brown adipose tissue (BAT), epididymal white adipose tissue (eWAT), and retroperitoneal white adipose tissue (rWAT). **D**) Intraperitoneal glucose tolerance test in control and *Sucnr1*-βKO mice (n=7-9). **E**) Oral glucose tolerance test in control and *Sucnr1*-βKO mice (n=3-4). **F**) Insulin tolerance test in control and *Sucnr1*-βKO mice. Data are displayed as mean ± SEM.

4.4.3. Impact of β-Cell-Specific SUCNR1 Ablation on *In Vivo* Diet-Induced Obesity and Insulin Resistance

In order to explore whether a diet-induced metabolic stress phenotype exists, we exposed control and *Sucnr1*-βKO mice to a high-fat diet (HFD). After 8 weeks of HFD feeding, no differences in body weight between the groups were observed (Figure R25A). Remarkably, *Sucnr1*-βKO mice showed an overall higher diet-induced hyperglycemia than control mice, which was particularly significant in random-fed conditions (Figure 25B). Simultaneously, *Sucnr1*-βKO mice showed lower plasma insulin levels, which were also significantly decreased in random-fed conditions (Figure 25C), suggesting a compromised β-cell function.

Examination of pancreatic islet morphology indicated a trend towards a lower β-cell mass in *Sucnr1*-βKO mice than in controls, without any evident changes in α-cell mass (Figure 25E). This observation suggests that SUCNR1 might be potentially in-

volved in β -cell mass regulation or survival, particularly under obesity compensation.

To probe whether specific β -cell disruption of *Sucnr1* affected glucose response in an obesity context, we performed glucose tolerance tests (GTTs) on mice subjected to HFD. To account for potential differences in incretin-induced responses in β -cells, we independently performed an intraperitoneal GTT (i.p. GTT) and an oral glucose gavage GTT (OGTT). Our strategy was influenced by our recent work identifying the intestine as a substantial contributor to circulating succinate^{342,347}, and our findings demonstrating an amplified succinate response to oral, as opposed to intravenous, glucose challenge in humans³⁵². In both tests, glucose tolerance was further impaired in *Sucnr1*- β KO mice compared to control mice, accompanied by a reduced insulin secretory response (**Figure 26A**). This outcome underlines SUCNR1's vital role in insulin secretion during glucose loading, regardless of the glucose administration route. Interestingly, these differences were not associated with changes in the glucose-associated GLP-1 response in *Sucnr1*- β KO mice on HFD (**Figure 26B**), further emphasizing the role of SUCNR1 in insulin secretion independently of the incretin pathway. Additionally, in assessing insulin sensitivity, we did not observe notable changes in *Sucnr1*- β KO mice on HFD. This was verified directly by an insulin tolerance test (**Figure 26C**) and indirectly by assessing the homeostasis model assessment index of insulin resistance (HOMA-IR) (**Figure 25D**), further supporting β -cell dysfunction –rather than an effect on peripheral tissues– eliciting the worsened glucose tolerance phenotype observed in *Sucnr1*- β KO mice.

Collectively, these results establish SUCNR1 function in β -cells as essential for insulin secretion within an *in vivo* obesity and insulin resistance context, as evidenced by the impaired glucose tolerance exhibited by *Sucnr1*- β KO mice on a high-fat diet.

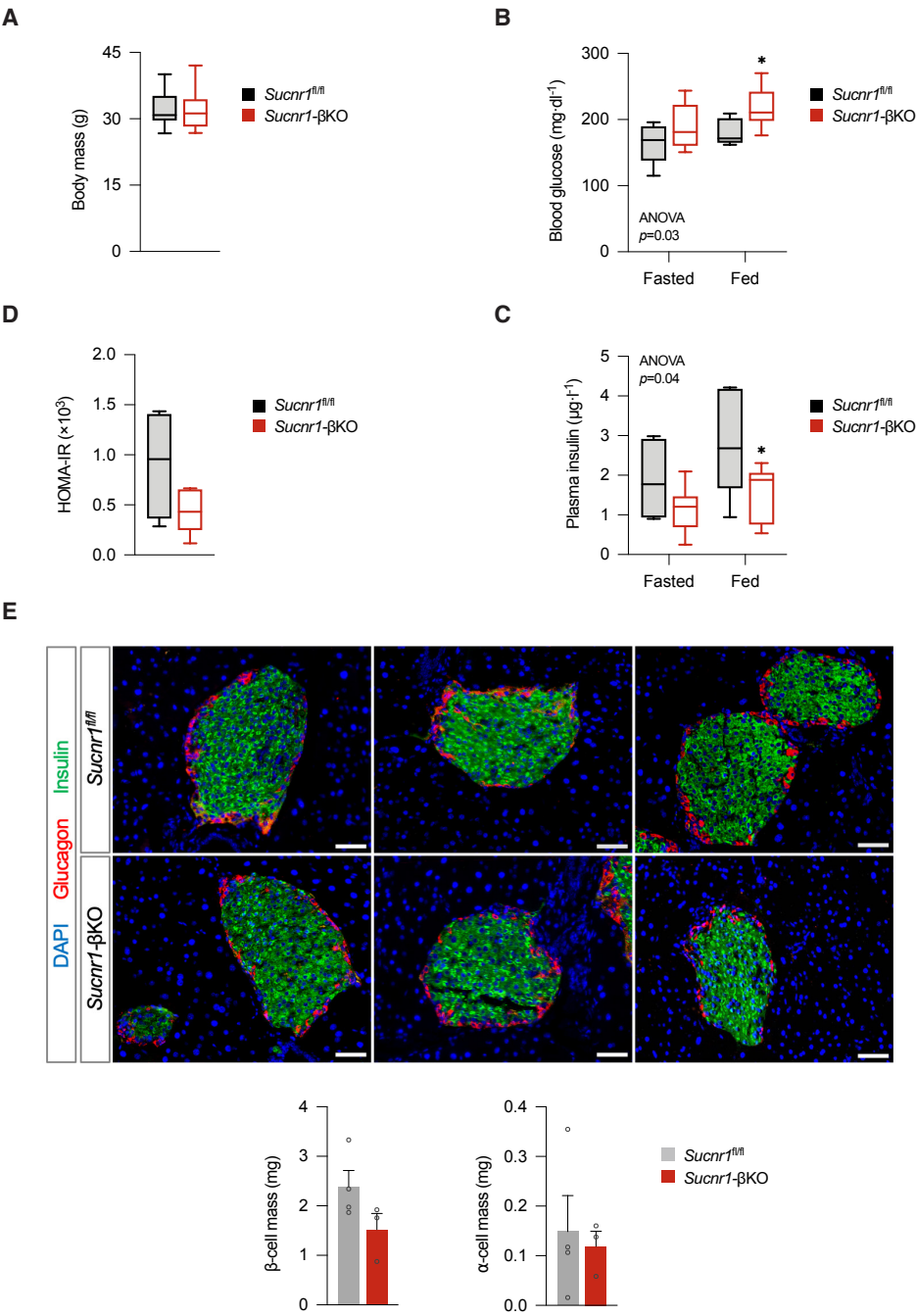
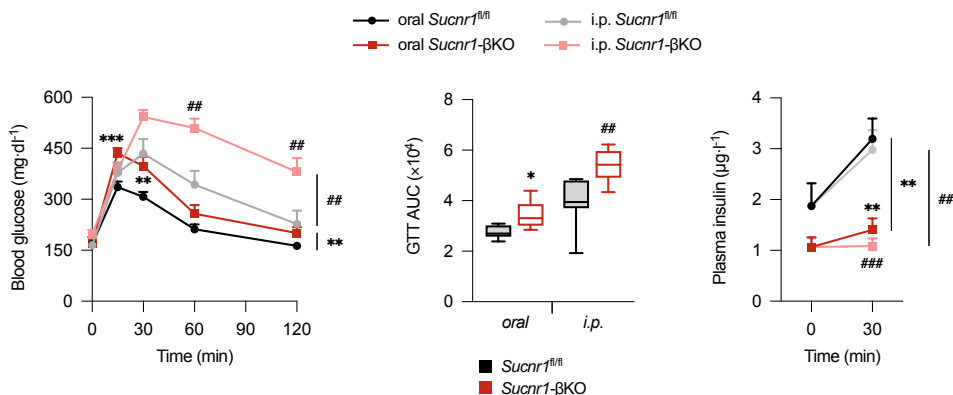
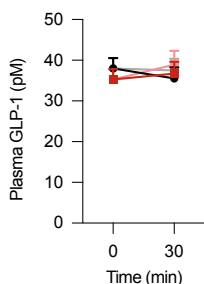


Figure 25. *Sucnr1*-βKO Mice on High Fat Diet (HFD). **A)** Body mass of control and *Sucnr1*-βKO mice under HFD for 8 weeks (n=8-9). **B)** Blood glucose levels in control and *Sucnr1*-βKO mice in fasted or random-fed conditions (n=7-8). **C)** Plasma insulin levels in control and *Sucnr1*-βKO in fasted or random-fed conditions (n=7-8). **D)** HOMA-IR index for control and *Sucnr1*-βKO mice (n=4-5). **E)** Morphometric analysis of control and *Sucnr1*-βKO mice by immunofluorescence staining with insulin and glucagon and counterstained with DAPI (n=3-4). Scale bar indicates 50 μm. Data are presented as mean ± SEM or as box-and-whiskers plots indicating the median, first and third quartiles, and maximum and minimum values. * $p<0.05$ vs. control mice (Student's t-test in **A**, **D**, and **E**, or two-way ANOVA with Bonferroni's test for multiple comparisons in **C** and **D**).

A



B



C

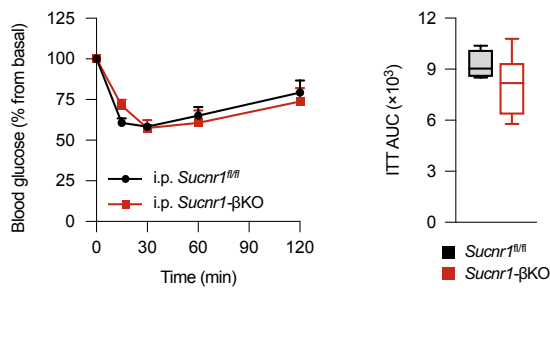


Figure 26. Metabolic Experiments on *Sucnr1-βKO* Mice on High Fat Diet (HFD). **A)** Intraperitoneal (i.p.) and oral glucose tolerance tests (GTTs) in control and *Sucnr1-βKO* mice (n=6-8) after 8 weeks of HFD feeding. Displayed are the blood glucose levels, the area under the curve (AUC), and plasma insulin (n=5-6). **B)** GLP-1 levels (n=5) during the glucose tolerance tests. **C)** Insulin tolerance test (ITT) in control and *Sucnr1-βKO* mice (n=6-7). Data are presented as mean ± SEM or as box-and-whiskers plots indicating the median, first and third quartiles, and maximum and minimum values. **p*<0.05, ***p*<0.01, ****p*<0.001 vs. control mice, or when comparing experimental groups in orally-administered mice; #*p*<0.05, ##*p*<0.01, ###*p*<0.001 when comparing experimental groups in i.p.-administered mice (Student's t-test when comparing two groups in AUCs in **A** and **C** or two-way ANOVA with Bonferroni's test for multiple comparisons in time-course assessments in **A-C**).

4.5. Study of Succinate Dynamics and Their Relationship with Insulin Secretion Potentiation in Humans

The significance of succinate in modulating insulin secretion has been emphasized through our work with preclinical models thus far. The subsequent phase of this thesis seeks to validate this role within the framework of human biology. Nonetheless, it is crucial to acknowledge the inherent challenges associated with studying β -cell function in live humans. These range from the inaccessible location of β -cells *in vivo* and their sensitivity to environmental alterations to the confounding influence of diverse factors such as age, diet, and lifestyle^{11,25,43}. This complexity, compounded by ethical considerations and the invasive nature of many research methodologies, underlines the value of mathematical modeling in this scientific exploration. The significance of these models lies in their ability to untangle individual factors' contributions and provide quantitative predictions under various physiological and pathological conditions, a task often impracticable through traditional experimental approaches^{215,465}. This is particularly relevant to the examination of *in vivo* β -cell function –a complex system in which metabolites, hormones, and intracellular signaling pathways coalesce to regulate insulin secretion¹¹. Mathematical modeling thus serves as a valuable tool in refining our understanding of succinate's role within human β -cell physiology.

To accomplish this, our strategy involved characterizing a human cohort of prediabetic subjects. The rationale behind this choice stems from the understanding that these individuals may display enhanced insulin secretion and potentially display distinct succinate dynamics in response to glucose challenges compared to normoglycemic subjects. Moreover, it is conceivable that the characteristic hyperinsulinemia observed in prediabetic patients could be linked to the succinate response in these individuals. Following the characterization of this cohort, we leveraged mathematical modeling to decipher the contribution of succinate within this specific context (**Figure 27**).

4.5.1. Profiling a Prediabetic Human Cohort for the Study of Succinate Dynamics

To gain insight into the potential relationship between circulating succinate kinetics and β -cell function, a meticulous characterization of a human cohort representing different glucose tolerance states was conducted. This cohort was composed of individuals classified as either normal glucose tolerant (NGT) or non-normal

Results

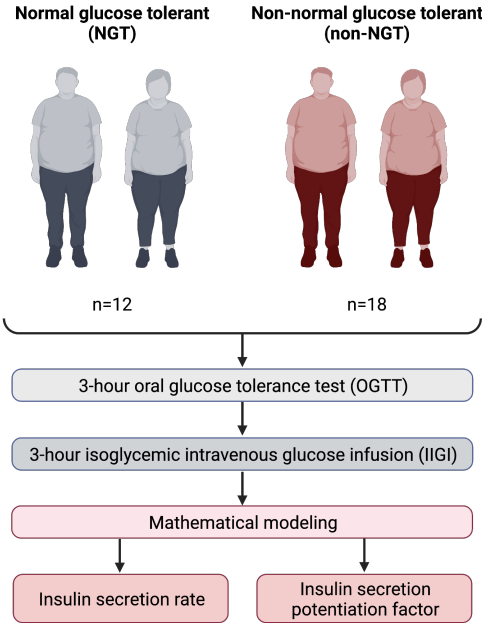


Figure 27. Schematic of the Study Cohort. Participants underwent specific procedures outlined in the study, and the data obtained from these procedures were utilized in mathematical modeling to evaluate β -cell function.

glucose tolerant (non-NGT). This classification was strictly based on the guidelines delineated by the ADA⁴²¹ (clinical and anthropometric data are detailed in [Table 4](#)). Accordingly, it should be noted that non-NGT individuals are considered prediabetic and exhibit impaired fasting glucose levels and/or impaired glucose tolerance as determined by the 2-hour OGTT, without meeting the full criteria for a diabetes diagnosis.

Two complementary approaches, the OGTT and the isoglycemic intravenous glucose infusion (IIGI), were employed to assess succinate’s role in β -cell function comprehensively. Using a carefully constructed algorithm, the IIGI was explicitly designed to mimic the plasma glucose profile observed during the OGTT for each study participant. When comparing identical plasma glucose concentrations, oral glucose stimulation, due to the incretin effect, elicits a greater insulin response than intravenous glucose administration. This effect is commonly quantified as the relative disparity between the insulin response to oral glucose versus isoglycemic intravenous glucose²¹³.

As expected, subjects in the non-NGT group had higher fasting plasma glucose and 2-hour glucose during the OGTT and displayed insulin resistance. Accordingly, these

patients exhibited greater glucose excursions during both the OGTT and IIGI (**Figure 28A**), which were concurrent with higher insulin (**Figure 28B**) and C-peptide responses (**Figure 28C**).

We found that circulating succinate presented a trend for higher fasting levels in the non-NGT group than in the NGT group (**Table 4**), in line with our previous findings indicating that circulating succinate serves as an early biomarker of metabolic dysfunction^{338,340}.

When assessing the response to the glucose challenges, no group differences were detected in the GLP-1 response (a recognized factor of the amplifying pathway)

Table 4. Clinical and Anthropometric Data of the Subjects Included in the Study

	Normal glucose tolerant (NGT)	Non-normal glucose tolerant (non-NGT)	p
n (female/male)	12 (9/3)	18 (8/10)	
Age (years)	36.2 ± 12.7	45.6 ± 11.0	0.039
BMI (kg·m ⁻²)	26.3 ± 2.8	30.5 ± 3.5	<0.001
Waist-to-hip (cm)	0.86 ± 0.07	0.94 ± 0.10	0.028
Fat mass (%)	31.5 ± 7.3	33.9 ± 6.8	0.373
FPG (mg·dl ⁻¹)	94.9 ± 4.8	106.0 ± 10.2	<0.001
Uric acid (mg·dl ⁻¹)	4.3 ± 1.0	5.8 ± 1.2	0.010
Total cholesterol (mg·dl ⁻¹)	155.8 ± 32.5	182.6 ± 36.4	0.049
HDL (mg·dl ⁻¹)	53.3 ± 10.1	51.7 ± 11.5	0.686
LDL (mg·dl ⁻¹)	86.5 ± 23.2	111.8 ± 32.0	0.026
Triglycerides (mg·dl ⁻¹)	80.4 ± 33.4	114.6 ± 64.6	0.084
AST (U·l ⁻¹)	17.8 ± 3.7	34.2 ± 22.4	0.002
ALT (U·l ⁻¹)	15.0 ± 4.3	27.2 ± 10.5	<0.001
GGT (U·l ⁻¹)	15.8 ± 7.0	58.9 ± 64.8	0.002
HbA1c (%)	5.1 ± 0.3	5.4 ± 0.3	0.005
2-h glucose (mg·dl ⁻¹)	120.4 ± 15.4	154.1 ± 40.5	0.003
Fasting insulin (pmol·l ⁻¹)	49.1 ± 14.6	64.9 ± 31.0	0.072
Fasting C-peptide (nmol·l ⁻¹)	0.33 ± 0.08	0.47 ± 0.19	0.030
Fasting GLP-1 (pmol·l ⁻¹)	24.0 ± 7.7	31.6 ± 17.7	0.121
Fasting succinate (μmol·l ⁻¹)	47.8 ± 22.6	60.8 ± 23.3	0.142
OGIS (ml·min ⁻¹ ·m ⁻²)	406.6 ± 30.7	331.3 ± 51.0	<0.001
Incretin effect (a.u.)	1.78 ± 0.66	1.43 ± 0.36	0.114

Data are presented as mean ± SD. p-values were determined by the Student's t-test (with Welch's correction if SD is inconsistent between groups) or the Mann-Whitney U-test according to the data distribution. BMI: body mass index; FPG: fasting plasma glucose; HDL: high-density lipoprotein; LDL: low-density lipoprotein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; HbA1c: glycated hemoglobin; GLP-1: glucagon-like peptide 1; OGIS: OGTT-derived insulin sensitivity index; a.u.: arbitrary units.

(**Figure 28D**). Nonetheless, the estimated total incretin effect measured by the OGTT-derived insulin sensitivity index (OGIS) was lower in non-NGT individuals (**Table 4**). Strikingly, however, the succinate response to glucose, which was found higher after nutrient passage through the gut (similar to GLP-1), was significantly higher in the non-NGT group than in the NGT group (**Figure 28E**).

The dynamic regulation of the succinate response to glucose challenges, as observed in both NGT and non-NGT groups, unveils a layer of intricacy in our understanding of glucose metabolism. This response corroborates our recent study documenting a similar dynamic reaction in healthy individuals³⁵². Strikingly, the non-NGT group, which exhibited an elevated insulin response during both glucose challenge tests, showed a notably amplified succinate response. This observation hints at a potential linkage between high succinate levels and enhanced insulin secretion. Given these intriguing findings, we harnessed a mathematical model in our next investigative stage. Using data from the current cohort study, the model was set to unravel a more profound and detailed understanding of succinate's potential role in insulin response regulation.

4.5.2. Harnessing Mathematical Modeling to Discern the Association of Succinate and Insulin Secretion Response in Humans

In this section, we leveraged mathematical modeling to discern the potential association between succinate and the human insulin secretion response. Various pertinent parameters to *in vivo* β -cell function, such as insulin secretion rate (ISR), β -cell glucose dose-response sensitivity, and insulin secretion potentiation effect, can be obtained by simultaneous modeling analysis of the IIGI and the OGTT data^{213,215,466}.

The ISR offers a nuanced understanding of the dynamic insulin response to glucose challenges. The conventional assessment of insulin secretion relies on deconvoluting C-peptide concentrations because, unlike insulin, it exhibits linear kinetics. This feature permits a direct correlation between C-peptide and insulin secretion, making it a valuable indicator for modeling the ISR^{213,466}. While fasting ISR did not demonstrate any pronounced differences between groups, a discernible upward trend was seen in the non-NGT group (**Figure 29A**). Interestingly, the stimulated ISR exhibited a heightened response in the non-NGT group during both OGTT and IIGI (**Figure 29B**). This observation aligns with the hyperglycemia and characteristic hyperinsulinemia typically associated with insulin-resistant states. These variations

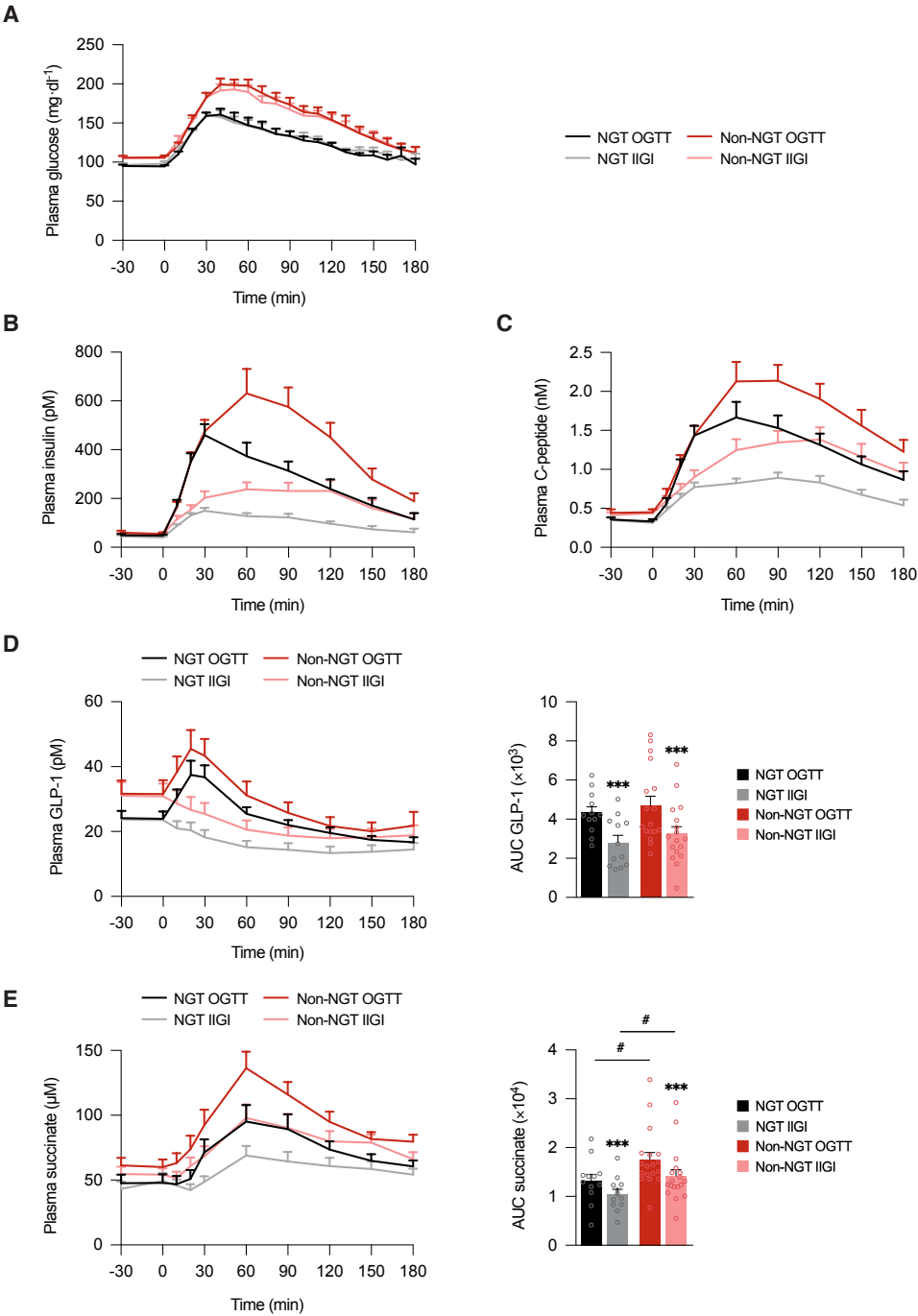


Figure 28. Assessment of Oral Glucose Tolerance Test (OGTT) and Isoglycemic Intravenous Glucose Infusion (IIGI) in Non-Normal Glucose Tolerant Subjects (non-NGT). Plasma **A)** glucose, **B)** insulin, **C)** and C-peptide levels during the OGTT and IIGI in normal glucose tolerant (NGT; n=12) and non-normal glucose tolerant (non-NGT; n=18) individuals. **D)** Plasma GLP-1 levels during OGTT and IIGI for both groups. **E)** Plasma succinate levels during OGTT and IIGI for both groups. Data are presented as mean ± SEM. ****p*<0.001 when comparing OGTT and IIGI; #*p*<0.05 when comparing NGT and non-NGT subjects (paired and unpaired Student's t-test, Mann-Whitney U-test or Wilcoxon test in **D** and **E**, according to the data distribution).

did not appear to be connected to changes in the estimated β -cell glucose sensitivity (**Figure 29C**), further emphasizing the multifaceted nature of glucose homeostasis.

The potentiation phenomenon, as described in prior research, enables the modulation of the glucose dose-response on insulin secretion. This process involves linking a β -cell model to a C-peptide kinetics model to predict C-peptide concentration based on glucose levels^{213,466}. In line with the GLP-1 response (**Figure 28D**), incretin potentiation was similar between groups during the OGTT ($p=0.7118$) (**Figure 29D**). Notwithstanding, during the IIGI, in which the incretin effect is considered negligible, the curve of glucose-induced potentiation described opposing dynamics between groups. IIGI potentiation exhibited a modest decrease in NGT individuals, while conversely, there was a slight increase in non-NGT individuals over time ($p<0.0001$) (**Figure 29E**). A correlation analysis was performed to further substantiate our hypothesis on succinate's role in insulin secretion potentiation, utilizing the potentiation ratio and the average succinate response during the IIGI. The results demonstrated a significant positive correlation (**Figure 29F**), suggesting a direct association between succinate levels and insulin secretion potentiation in humans.

Altogether, these findings underscore the impact of succinate as an amplifier of insulin secretion in humans, a process potentially crucial in circumstances of hyperinsulinemia often noted in people with prediabetes.

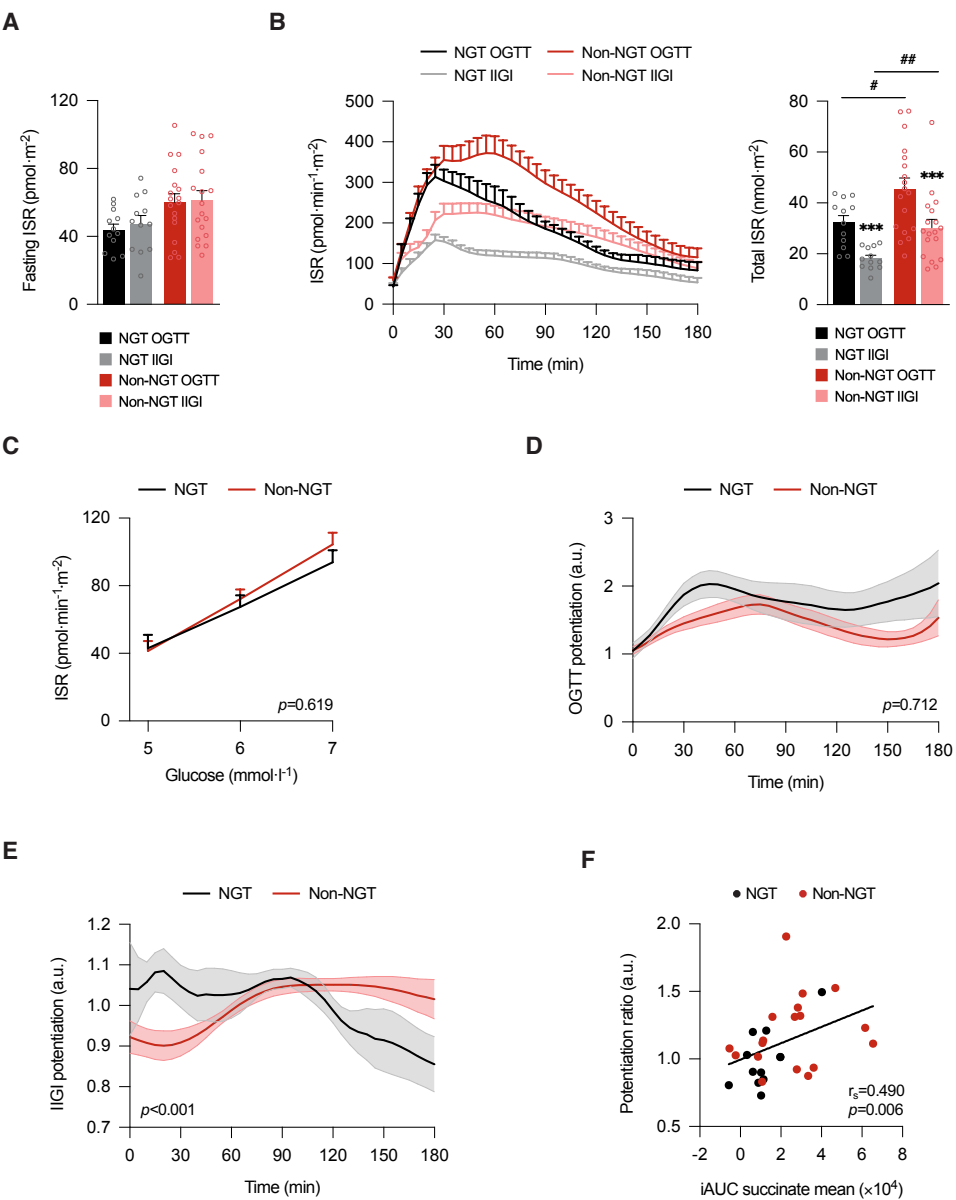


Figure 29. Mathematical Modelling Employment for the Analysis of Succinate Dynamics on Insulin Secretion Potentiation in Humans. **A)** Fasting insulin secretion rate (ISR) in normal glucose tolerance (NGT; n=12) and non-NGT groups (n=18). **B)** Total ISR during oral glucose tolerance test (OGTT) and isoglycemic intravenous glucose infusion (IIGI) in NGT and non-NGT groups. **C)** β -cell glucose sensitivity represented as a dose-response function between ISR and glucose concentrations for both groups. **D)** Incretin-related potentiation was calculated during OGTT in relation to IIGI for both groups. **E)** Potentiation calculated during IIGI for both groups. **F)** Correlation between the potentiation factor mean and the incremental area under the curve (iAUC) of succinate during the IIGI for both groups (n=30). Data are presented as mean $\pm$ SEM. *** $p<0.001$ when comparing OGTT and IIGI; # $p<0.05$, ## $p<0.01$, when comparing NGT and non-NGT subjects (paired and unpaired Student's t-tests in **A** and **B**, Mann-Whitney U-test in **B**, Wilcoxon test in **B**, two-way ANOVA tests in **C-E**, or Spearman's rank correlation coefficient in **F**).

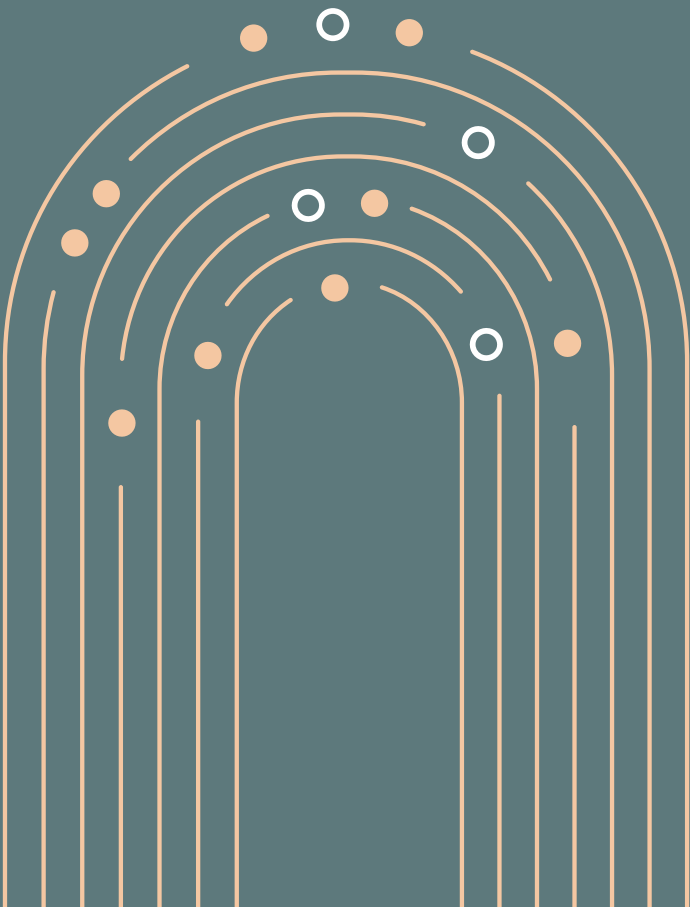
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UNCOVERING NOVEL INSIGHTS INTO INSULIN SECRETION REGULATION AND DIABETES PATHOGENESIS

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Discussion

5



UNIVERSITAT ROVIRA I VIRGILI

THE SUCCINATE-SUCNR1 AXIS AND ITS IMPACT ON BETA-CELL FUNCTION: AN INTEGRATIVE STUDY
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The centrality of β -cells in physiology and diabetes can hardly be overemphasized. Representing the cornerstone of metabolic control in the body, these specialized cells nested within the pancreatic islets of Langerhans are primarily responsible for insulin production and secretion in response to glucose and other nutrient surges. This unique role places the β -cells at the crux of maintaining glucose homeostasis, ensuring that the body's tissues have access to the correct quantity of glucose necessary for their function^{11,25,30}. However, the pivotal role of β -cells extends beyond this glucostatic function, implicating their overall health and optimal performance in broader physiological realms, including lipid metabolism, satiety signaling, and even cardiovascular function through additional insulin actions^{90,467}.

As the β -cell's function and overall health diminish, the shadow of diabetes emerges¹³⁸. It is estimated that most of the β -cell function is already lost at the onset of T2D, a pandemic that, alarmingly, is on a precipitous rise globally^{14,15}. The expanding prevalence of T2D, marked by progressive β -cell dysfunction and failure, is accompanied by devastating health complications such as kidney disease, retinopathy, neuropathy, and an increased risk of cardiovascular disease^{138,153,468}. The significant health, social, and economic impact of this global epidemic underscores the urgent need for scientific research to unravel the intricate mechanisms underlying β -cell functionality and dysfunction.

One area that has recently piqued scientific interest is the role of the succinate-SUCNR1 axis, not only in disease but also in physiology. Succinate, a TCA cycle intermediate, is increasingly being recognized as more than just an energy bystander but a signal molecule with significant implications for cellular physiology and pathophysiology²⁷². Through its receptor, SUCNR1, succinate has been implicated in a plethora of pathological conditions, including hypertension, ischemia-reperfusion injury, inflammation, and metabolic diseases like obesity^{272,393,395,408,469}. However, the role of the succinate-SUCNR1 axis has only been recently started to be studied in physiological responses, where it has demonstrated to be crucial in anti-inflammatory actions^{395,397}, adipose tissue function⁴¹², and muscle physiology³²⁶.

In this doctoral thesis, we navigated into the uncharted link between the succinate-SUCNR1 axis and β -cell function. While previous research in this area primarily focused on the ability of cell-permeable succinate analogs to stimulate insulin secretion through the TCA cycle and anaplerosis^{66,315,316}, our findings reveal that beyond its intracellular functions, extracellular succinate stimulates insulin secretion in hyperglycemic states in mice and humans *via* SUCNR1 activation. This mecha-

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nism represents a novel amplifying pathway for insulin secretion, which may be relevant in prediabetic states where the oversecretion of insulin is an adaptive response to overcome insulin resistance.

5.1. Selective and Metabolic-Dependent Regulation of SUCNR1 Expression in Islets and β -Cells

Various GPCR-mediated mechanisms collaborate in β -cells to orchestrate insulin secretion⁴¹⁶. Among these, SUCNR1 has emerged from relative obscurity. Before our research, this GPCR was uncharted territory in the landscape of pancreas biology. As a foundation, we examined SUCNR1 expression across an array of metabolic tissues in mice, finding consistency with prior research^{445–447} in confirming the presence of *Sucnr1* mRNA within the pancreas, although it appears to be lower compared to other tissues such as WAT. Indeed, the relatively low abundance of GPCR levels compared to many other cellular receptors and their associated high Ct values often present a hurdle in detecting them. Consequently, they are frequently bypassed in relative gene expression and omics analyses⁴⁷⁰, underscoring the challenges entailed in SUCNR1 studies.

Intriguingly, our qualitative results unveiled a differential prevalence of SUCNR1 protein between pancreatic islets and exocrine tissues, showcasing a unique distribution pattern. Furthermore, the relative abundance of SUCNR1 within islets highlighted a distinctive molecular feature potentially capable of influencing islet physiology.

Building upon this expression profile, our data underscore the specific cellular domain of SUCNR1 within islets. Examining *Sucnr1* gene expression levels among the two major islet cell populations (i.e., α - and β -cells) illustrated its primary expression in β -cells derived from rat islets. This newfound aspect contributes to our comprehension of SUCNR1, shedding light on its potential implications for β -cell function.

Additionally, our computational study on the human SUCNR1 gene employing the Islet Regulome Browser⁴⁴¹ has pinpointed NKX2.2, FOXA2, and NKX6.1 as plausible controllers of *SUCNR1* expression. These transcription factors are known to be critical determinants of β -cell identity and function^{448–450}. This highlights the potential significance of SUCNR1 in sustaining and regulating β -cell function, thus underlining the necessity to delve deeper into its role in this domain.

The investigation into glucose-mediated alterations in succinate release and SUCNR1 expression has unveiled novel insights. While hyperglycemia has been previously associated with intracellular succinate accumulation in β -cells³²³, the present research is –to our knowledge– a pioneering effort in quantifying extracellular succinate in β -cells following glucose stimulation. In this particular situation, an amplified succinate release under high-glucose conditions has been noted in mouse β -cells, chiefly driven by glucose catabolism via the TCA cycle³¹⁸. This newly discovered information might posit a potential autocrine regulation pathway in β -cells. According to this mechanism, exposure to glucose could induce a rise in succinate accumulation, which is subsequently exported, potentially expediting SUCNR1 activation.

The dynamic modulation of GPCR expression in β -cells bears significant implications for cell functionality, particularly in disease states such as diabetes⁴⁷¹. Notably, our study revealed a dose-responsive increase in *Sucnr1* expression aligned with varying glucose concentrations in mouse β -cells, which was corroborated at the protein level in the human β -cell line EndoC- β H1. This fluctuating and adaptative expression, dictated by glucose levels, highlights the nuanced interaction between SUCNR1 and glucose homeostasis in the context of β -cell biology.

Upon further exploration, we detected a substantial increase in SUCNR1 expression in islets procured from donors with obesity and T2D compared to those from healthy controls. This observation converges with our previous findings of elevated circulating succinate levels in the former groups^{338,340}. These data suggest that a surge in metabolic load, as reflected by a higher donor BMI, may trigger an increase in SUCNR1 expression in islets, which can be perceived as a potential adaptive response of β -cells to metabolic stress.

In particular, differential GPCR expression like GIPR and GLP-1R in β -cells is not only crucial for normal cell function but also demonstrates altered patterns in pathological states. Accordingly, chronic hyperglycemia, as observed in diabetes, has been reported to downregulate these receptors, leading to impaired β -cell functionality and insulin secretion^{471–473}. However, our findings in human islets and β -cell models revealed an upregulation of SUCNR1 in association with hyperglycemia, which contrasts with the reported downregulation of GLP1-R under high-glucose conditions^{472,473}. This hints at a compensatory mechanism in which SUCNR1 may continue, or attempt to maintain its functionality, during the progression of T2D.

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Remarkably, the *in silico* *SUCNR1* expression analysis further revealed that several identified risk variants for T2D have been mapped at the *SUCNR1* locus or are close to its *cis*-regulatory elements^{441,449,474}. This could imply that genetic predisposition towards alterations in *SUCNR1* expression or function might potentially have an influence on the etiology of T2D.

Collectively, the aforementioned discoveries indicate a prospective association between alterations in *SUCNR1* expression and activity within β -cells during specific pathological conditions, which could contribute to metabolic adjustments at the cellular level and subsequently increase the susceptibility to the onset and the progressive worsening of diabetes over time.

5.2. Extracellular Succinate Sensing by β -Cells and Subsequent Insulin Release *via* a *SUCNR1*-Dependent Mechanism

The amplifying pathway, an intricate aspect of β -cell physiology that modulates insulin secretion, remains partially obscure; however, the present study may have provided significant insights into this intricate mechanism. The conventional model of glucose-induced insulin release from β -cells establishes a sequence where glucose metabolism increases the ATP/ADP ratio, resulting in the closure of K_{ATP} channels. This leads to β -cell depolarization, the opening of voltage-dependent Ca^{2+} channels, an influx of Ca^{2+} , and insulin granule exocytosis¹¹. However, this sequence, known as the triggering pathway, accounts for only a fraction of the insulin release in response to glucose. The remaining portion, referred to as amplifying pathways, is independent of K_{ATP} but responds to metabolic and extracellular signals and is estimated to account for as much as 60-70% of insulin secreted in response to glucose stimulation⁸². By identifying extracellular succinate as a potential amplifying signal through *SUCNR1*, our findings shed light on this area and may offer inspiration for further research and therapeutic strategies in diabetes management.

The present study underscores the significant *SUCNR1* contribution in β -cells, enhancing insulin secretion in response to elevated glucose levels. Notably, this function exhibits similarities to incretins such as GLP-1 and GIP⁴⁷⁵. However, the precise glucose-dependent mechanism through which incretins influence β -cells is yet to be fully elucidated. Investigations into this issue have yielded significant insights. One study reported that metabolic coupling through cytosolic glutamate –a by-

product of the malate-aspartate shuttle during glucose metabolism– is essential for the downstream cAMP-PKA effect that incretin receptors impose on insulin secretion⁴⁷⁶. Another important discovery comes from a study identifying the role of Gs/Gq switching in incretin signaling during acute versus chronic hyperglycemia⁴⁷⁷.

In this context, we observed that MIN6 cells did not react to extracellular succinate concerning Ca^{2+} mobilization when subjected to a low glucose concentration. Conversely, when exposed to high glucose levels, these cells exhibited a significant Ca^{2+} response to extracellular succinate, indicating a glucose-dependent mechanism. This observation aligns with our GSIS results in mouse and human β -cells, where SUCNR1 activation predominantly functions as an insulin secretagogue under high glucose conditions. The question remains as to whether SUCNR1 operates through similar mechanisms to incretin receptors to stimulate insulin secretion in β -cells, which is an area that calls for additional exploration.

Regarding the transduction pathways underlying this insulinotropic effect, SUCNR1 signaling differs among cell types⁴⁶¹ but with a preference to engage Gi or Gq proteins^{385,397}. Here, we identified a Gq- and PKC-dependent mechanism linking SUCNR1 activation to the potentiation of insulin secretion. While some research has speculated that increases in intracellular Ca^{2+} observed upon SUCNR1 activation are mediated by Gi and $\beta\gamma$ -subunit-induced PLC- β activation rather than Gq^{478,479}, recent studies have confirmed the necessity of Gq engagement for PKC- and Ca^{2+} -mediated responses to SUCNR1 activation in immune cells^{397,480}. Likewise, other GPCRs, such as FFAR1 (activated by free fatty acids), have been reported to use pathways mediated by Gq, PKC, and Ca^{2+} , which are known to stimulate insulin secretion^{481,482}.

We further explored the activation of SUCNR1, which elicited differential activation of several proteins instrumental in the induction process. For instance, PKC δ and PKC λ/ζ , from the novel and atypical PKC classes, respectively, were observed to activate shortly after SUCNR1 activation in β -cells. These proteins play a significant role in β -cell function, regulating insulin secretion, with PKC ζ specifically being essential for β -cell replication during compensatory hyperglycemia⁴⁸³. We also identified Akt as an activated target upon succinate or cESA stimulation in β -cells. This protein kinase is crucial in multiple signaling pathways, relevant for cell survival in β -cells⁴⁸⁴.

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Furthermore, in the downstream signaling mediated by SUCNR1, we noted that GSK-3 α/β activity was blocked (by inhibitory serine-phosphorylation) following receptor agonism. A decrease in GSK-3 activity has been linked to improved β -cell turnover and function, while its enhanced activity is associated with β -cell failure and reduced proliferation and mass⁴⁸⁵. In the MAP kinase family, we observed that ERK1/2 was activated, which has been shown to provide protection against apoptosis in β -cells and to promote insulin exocytosis^{486,487}. Concurrently, p38 was inactivated following SUCNR1 stimulation, which p38 inhibition in β -cells is associated with enhanced insulin secretion and prevention of islet apoptosis^{488–490}. However, this decrease in p38 activity upon a GPCR activation is atypical, as a canonical signal often involves increased p38 phosphorylation⁴⁹¹. Further research is required to discern whether SUCNR1 genuinely mediates this effect in β -cells or whether it results from the basal status of the employed cell line.

Through our SUCNR1 signaling study, we also observed an increase in CREB activity upon receptor agonism. This CREB activation, driven by cAMP and Ca²⁺, rejuvenates β -cells, rendering them metabolically primed for the subsequent feeding cycle^{492,493}. Notably, CREB is significant in promoting β -cell survival and proliferation *in vivo*. Any dysfunction in CREB activity can lead to apoptosis, glucose intolerance, and, ultimately, the development of T2D^{494–496}.

Overall, our research unveils the critical role of the succinate-SUCNR1 axis as a pioneering amplifying pathway for insulin secretion under hyperglycemic conditions. Specifically, we provide evidence that SUCNR1 activation in β -cells mediates a Gq and PKC signaling mechanism involving Ca²⁺ mobilization, culminating in an enhancement of insulin release under high glucose concentrations.

5.3. Impact of Specific β -Cell SUCNR1 Ablation in *In Vivo* β -Cell Function and Glucose Tolerance

SUCNR1 activation is commonly recognized as a mechanism bolstering tissue damage^{355,383,394,405,497,498}. Indeed, chronic elevation of extracellular succinate is found in low-grade inflammatory, metabolic disorders such as cardiovascular disease, obesity, and T2D^{335,338,339,356}. However, circulating succinate levels also rise transiently in response to normal physiological processes such as cold exposure³³⁶, exercise^{326,348}, and food intake³⁵². Furthermore, the nutritional regulation of succinate depends on glucose sensing by the intestine and the metabolic status of the subject³⁵², suggesting a key signaling role for succinate for metabolic processes related to energy manage-

ment²⁷². Along this line, our group recently showed that the succinate-SUCNR1 axis is a novel regulator of leptin production in adipocytes⁴¹². Building upon our current findings, we have discovered an exciting new function for extracellular succinate: it acts as an insulin secretagogue. Moreover, this study provides robust evidence supporting the inclusion of SUCNR1 as a crucial component of the cellular metabolite-sensing machinery, enabling cells to maintain energy balance effectively.

Consistent with this hypothesis, we show that β -cell specific depletion of SUCNR1 in mice leads to an aberrant metabolic phenotype characterized by increased glucose intolerance and defects in insulin secretion under HFD feeding. Interestingly, we observed a more significant disparity in glucose intolerance among mice administered intraperitoneally, suggesting the indispensable role of endogenous glucose metabolism and subsequent succinate production in governing SUCNR1's functionality within β -cells.

As no significant variations in β -cell mass were detected, SUCNR1 may primarily influence the functionality of adult β -cells rather than in their development or the compensatory changes in β -cell mass that occurs during obesity⁴⁹⁹. However, *Sucnr1*- β KO mice on an HFD exhibited a trend towards the reduced β -cell mass. While this observation may not be definitive, it warrants a more in-depth investigation into the role of SUCNR1 in β -cell proliferation and apoptosis to understand better its potential influence on β -cell mass adjustments in obesity and T2D.

The observed similarities in the GLP-1 response between *Sucnr1*- β KO mice and control mice on an HFD, coupled with the pronounced impairment in insulin secretion and glucose tolerance in the *Sucnr1*- β KO mice, underscores the pivotal role of SUCNR1 in preserving insulin secretion. Despite the absence of incretin response both in *Sucnr1*- β KO mice and control mice in diet-induced obesity, β -cells in *Sucnr1*- β KO mice fall short in counteracting hyperglycemia, which emphasizes the critical involvement of the succinate-SUCNR1 axis in this scenario. Moreover, our findings align with an earlier study using global *Sucnr1* knockout mice, which exhibited progressive glucose intolerance under HFD and impaired insulin response as part of a complex phenotype⁴⁰⁸. However, other organs (e.g., adipose tissue) may also influence these phenotypes, underscoring the importance of analyzing the effects of SUCNR1 in specific cell types^{272,395,412}.

5.4. Succinate's Role as a Hormone and Its Association with Enhancing Insulin Secretion in Humans

Commonly, tissue extracellular succinate concentrations increase in response to acute physiological stressors such as tissue hypoxia and inflammation^{279,280}. Meanwhile, emerging research indicates that transient surges in succinate levels are not just a mere consequence but could be crucial in facilitating proper physiological responses²⁷². However, a starkly different pattern emerges in the context of metabolic disease. In conditions like obesity and diabetes, circulating succinate levels remain chronically elevated, often signaling a poor prognosis^{338,340}. Additionally, the modulation by food ingestion usually observed in healthy individuals becomes blunted under these conditions³⁵². This implies that the intricate regulation of succinate is disrupted during metabolic dysfunction, leading to a distorted signaling system.

Our study in patients highlights the significance of the succinate-SUCNR1 axis in preserving glucose homeostasis during metabolic challenges. Prediabetes refers to a condition where glucose intolerance and elevated basal glucose values are below the diabetes threshold. This usually occurs alongside peripheral insulin resistance, which is counterbalanced by increased secretion of insulin⁵⁰⁰ through mechanisms that are still not fully understood. When challenged with glucose, we found that people with prediabetes exhibited an amplified succinate response, whereas their GLP-1 response mirrored that of subjects with normal glucose tolerance (NGT). These findings suggest that factors beyond incretins might play a role in modulating the heightened insulin secretion observed in prediabetic conditions.

No significant differences in incretin-related potentiation were observed between the groups during an OGTT, likely due to preserved operation of the incretin system in patients with non-NGT. However, during intravenous glucose infusion, where the incretin effect on insulin secretion is negligible⁹⁷, we found a positive correlation between the succinate response and insulin secretion potentiation. In this scenario, succinate takes on a hormone-like function, emerging as a potential novel regulator of the observed hyperinsulinemic response in prediabetic patients.

The succinate response to glucose is higher during –but not exclusive to– the oral challenge³⁵², which prompts the consideration of other succinate sources besides the intestine. We observed that β -cells secrete more succinate when exposed to high glucose, consistent with previous studies showing that hyperglycemia can also lead to local increases in succinate^{391,498}, likely due to overactivation of the TCA cycle⁵⁰¹.

Thus, it is tempting to speculate that endogenously produced succinate in β -cells could be partly responsible for SUCNR1 engagement in an autocrine fashion, aligning with our *in vitro* observations. Succinate derived from the gut microbiota is also a potential source²⁷², and indeed other metabolites derived from the gut microbiota, including short-chain fatty acids, have been described as insulin secretagogue GPCR ligands^{502,503}.

Consequently, succinate is proposed as a potential novel regulator of the compensatory hyperinsulinemic response in people with prediabetes. Moreover, the interconnected roles of succinate in cellular metabolism, signaling, and its influence on insulin secretion potentiation further demonstrate the complexity of this intriguing metabolite.

5.5. Significance of the Study and Future Directions

In the present thesis, we employed an integrative strategy, harnessing the power of *in vitro* β -cell models, a unique β -cell specific SUCNR1 KO mouse model, and a human cohort encompassing people with both NGT and prediabetes. This combination of diverse methodological approaches, each offering unique and complementary insights, has coherently substantiated our central assertion. We can confidently state that succinate, *via* its interaction with SUCNR1, potentiates insulin secretion during episodes of hyperglycemia (**Figure 30**). By examining the succinate-SUCNR1 axis, our study becomes a pivotal component in the wider scientific discussion surrounding the regulation of physiology and metabolism. Furthermore, the implications of our findings are substantial, especially considering the escalating prevalence of metabolic diseases, including T2D.

Within this framework, our findings highlight SUCNR1 as a potential new therapeutic target to enhance insulin secretion in the context of T2D development. Indeed, the nutritional modulation of succinate is lost in patients with morbid obesity and T2D³⁵². Nonetheless, further research is needed due to the broad expression of SUCNR1 and its pleiotropic functions²⁷². While the succinate-SUCNR1 axis is crucial for the resolution of inflammation^{395,397}, activation of SUCNR1 in an inflammatory context could be detrimental due to its pro-inflammatory effects in the acute phase of the immune response^{394,504}. Therefore, directly targeting SUCNR1 to boost insulin secretion in T2D patients may not be the most effective course, given the receptor's diverse roles and potential off-target impacts. Along these lines, mitigating the higher levels of circulating succinate acting on its intestinal source, rather than di-

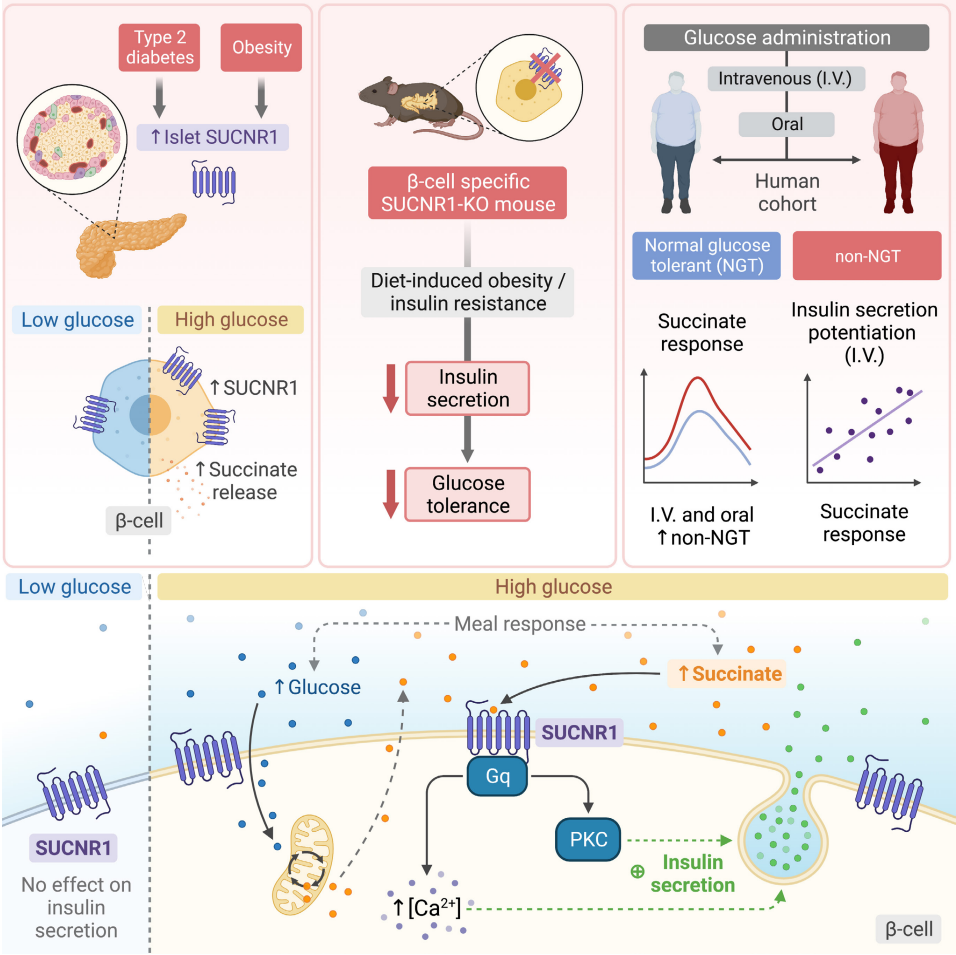


Figure 30. Succinate-SUCNR1 Interaction in β-cells Potentiates Insulin Secretion: Evidence from the Integrative Study. This figure illustrates the comprehensive experimental approach adopted in this study. It demonstrates the application of *in vitro* β-cell models, a β-cell specific SUCNR1 knockout (KO) mouse model, and a human cohort comprising individuals with normal glucose tolerance and prediabetes. Integrating these complementary methodologies confirms the thesis’ central hypothesis: the interaction between succinate and the SUCNR1 receptor within β-cells intensifies insulin secretion in response to hyperglycemia.

rectly activating SUCNR1, has recently emerged as a potential strategy for treating metabolic diseases⁵⁰⁵. Whether this strategy could also be effective in recovering the function of SUCNR1 on insulin secretion deserves further analysis.

While revealing, our study presents limitations and poses additional questions for future exploration. For instance, the established connection between succinate and the enhancement of insulin secretion across multiple models could be reinforced with additional *in vivo* evidence. Detailed clamp studies with exogenous succinate or cESA could provide further substantiation; however, the complexities and challeng-

es associated with such studies should be recognized. In comparison, experiments involving the administration of SUCNR1 agonists to both control and *Sucnr1*- β KO mice to assess their influence on insulin secretion emerge as a more feasible alternative in this context and would provide more translatable results. Nevertheless, the potential for unfavorable pleiotropic effects arising from systemic SUCNR1 activation^{272,457,469,480,506} could limit the efficacy of these approaches.

Drawing on our observations from IHC staining and gene expression analysis in isolated rat islet cell populations, we reasonably inferred that the increased SUCNR1 levels observed in islets from patients with obesity and T2D likely originate from β -cells. However, we did detect a slightly positive signal in the exocrine compartment of the pancreas, indicating potential expression in acinar or ductal cells. While our findings point towards β -cells as the primary source of differential SUCNR1 expression in islets, our conclusions are currently limited by the absence of available anti-SUCNR1 antibodies suitable for immunofluorescence. This technique could have allowed us to more definitively confirm SUCNR1 expression in all cell types within the pancreas by hormone or specific marker co-staining. In addition, future studies employing techniques such as RNA fluorescent *in situ* hybridization could provide a more robust understanding of SUCNR1's expression pattern and confirm or potentially refute the unique expression of SUCNR1 within β -cells.

Interestingly, our research intersects with a recent work highlighting an association between SUCNR1 polymorphisms and susceptibility to T2D and hypertension⁵⁰⁷. This association echoes our *in silico* study's results, where GWAS data from T2D patient populations revealed potential SNPs associated with a higher risk of T2D. Notwithstanding, we must bear in mind that genetic association studies, despite their valuable insights, cannot independently establish a causal link. Pursuing additional experimental studies to replicate these genetic variants in animal or cell models could be highly informative. These studies might enable a more detailed understanding of how these mutations impact SUCNR1 gene expression or protein function. Ultimately, such findings could illuminate novel aspects of T2D pathogenesis and progression, subsequently aiding the design of innovative therapeutic strategies.

Our research scrutinized the distinct expression patterns and downstream signaling of SUCNR1 and its subsequent impact on β -cells. However, it is important to acknowledge that the function of GPCRs like SUCNR1 can be modulated by mechanisms that extend beyond those hereby examined, including β -arrestin signaling –a critical process that both desensitizes the receptor by blocking further G protein

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activation and simultaneously initiates alternative signaling pathways⁵⁰⁸. Other regulatory mechanisms comprise receptor trafficking between the plasma membrane and intracellular vesicles, receptor degradation mediated by ubiquitination, and biased receptor agonism^{375,381,509}. An expanded investigation into these processes could uncover whether SUCNR1 is chiefly situated in the plasma membrane or resides within intracellular vesicles primed for exposure to the extracellular surface of β -cells in response to glucose. Moreover, such an exploration could elucidate the conditions or triggers that govern the rate of SUCNR1 internalization. It may also shed light on the intricacies underlying the observed subtle differences in the temporal activation of downstream mediators after SUCNR1 stimulation by cESA compared to succinate. Through this examination, we may obtain valuable insights into the factors potentially impacting β -cell sensitivity to extracellular succinate and SUCNR1 activity.

Moreover, the observed involvement of SUCNR1 activation with an array of intracellular signaling proteins (including PKCs, Akt, ERK1/2, CREB, p38, and GSK-3) alludes to its multifaceted role in β -cell activities, reaching beyond the confines of the presently investigated acute receptor activation in *in vitro* assays. This pivotal network could potentially influence a wide range of cellular processes, such as β -cell proliferation and protection against metabolic stressors found in obesity and T2D. Additional possibilities might encompass the regulation of β -cell maturation, their adaptive response to varying glucose levels, and the modulation of the immune response within the islet microenvironment^{484,485,489,490}. However, the full extent of these prospective roles necessitates a more comprehensive exploration in future research.

To explore the implications of absent SUCNR1 signaling in β -cells, our research primarily centered on *in vivo* studies using mice and human *in vitro* models. While this approach has yielded important insights, the absence of *ex vivo* islet functional analyses does present a limitation worth acknowledging. Although not designated as essential for our primary research scope, conducting *ex vivo* GSIS assays with both control and *Sucnr1*- β KO-derived mouse islets could have further enriched our comprehension of the phenomena under study. Specifically, this would allow us to evaluate the precise effects of varying glucose levels on insulin secretion, especially considering the observed influence of diet-induced obesity in *Sucnr1*- β KO mice. Additionally, integrating gene and protein expression analyses, or even unbiased omics studies such as RNA-seq, in these islets or isolated β -cells could provide deeper insights into the molecular mechanisms at play. Eventually, such approaches could

uncover potential alterations and clarify any long-term implications resulting from the absence of SUCNR1 signaling.

Historically, studies examining intracellular succinate's role in islets have relied on permeable succinate esters^{66,318}. These analogs are primarily effective in non-murine models attributed to the lack of malic enzyme activity –a link between succinate metabolism and insulin release *via* NADPH production– in mouse islets³¹⁸. The present study, however, raises a compelling question: might succinate uptake in human β -cells occur and exert significant effects? According to our findings, the capacity of extracellular succinate to stimulate insulin secretion appears to be primarily mediated through SUCNR1. This conclusion is supported by using a specific SUCNR1 antagonist in our experiments.

Nonetheless, we made an intriguing observation that under no-glucose conditions, insulin release was elicited by simultaneous SUCNR1 inhibition and extracellular succinate exposure, indicating a potential residual succinate uptake into β -cells and a further mechanism amplified by the lack of SUCNR1 signaling.

Nonetheless, we made an intriguing observation that SUCNR1 inhibition, in conjunction with extracellular succinate exposure, triggered insulin release in conditions devoid of glucose. This suggests that the lack of SUCNR1 signaling might induce insulin secretion in no-glucose states, and this mechanism might be further sustained by succinate uptake. In this context, building upon recent findings on cancer cells, which have demonstrated an ability to modulate mitochondrial function *via* SUCNR1⁵¹⁰, it is reasonable to propose that the absence of autocrine SUCNR1 activation could result in an augmented TCA cycle rate. This, in turn, might facilitate an increase in succinate production and, subsequently, insulin secretion in β -cells, which might only be noticeable in the absence of glucose. This hypothesis may offer a novel perspective on the role of succinate and SUCNR1 in the complex machinery of metabolic insulin secretion regulation, warranting further investigation.

As we continue to unravel the intricate roles of succinate within biological systems, a promising area of research presents itself in the form of examining succinate transport across cells and, particularly, understanding the potential impact of local succinate concentrations on autocrine and paracrine signaling in β -cells. A key question arises from our observation that *in vitro* β -cell models necessitate elevated succinate concentrations for SUCNR1 activation compared to the concentrations typically observed in circulation following meal intake or glucose administration. Specifically, succinate plasma concentrations fluctuate between 40-150 μ M during postprandial

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states, as supported by our prior research³⁵² and the present study. Nevertheless, human β -cells seem to respond at a higher level of 500 μ M. This finding might suggest a higher EC_{50} for SUCNR1 in β -cells compared to the estimates for other cell types (approximately 20-50 μ M)²⁷². A clarification of these results could help determine whether succinate produced by β -cells or islets leads to localized spikes in succinate concentrations and could bolster our hypothesis concerning the critical role of autocrine/paracrine signaling in the regulation of the succinate-SUCNR1 axis.

Incorporating the latest insights on succinate dynamics in muscle tissue³²⁶, we could postulate a model for islet cells where intracellular pH might govern succinate's release, thus using succinate to communicate local energetic status within islets. Analogous to the muscle context, this exported succinate could then bind to SUCNR1 receptors in nearby β -cells, facilitating a mode of autocrine or paracrine signaling that might be pivotal for the succinate-SUCNR1 axis function on insulin secretion. Indeed, considering the systemic impact of succinate on processes like energy metabolism, hypoxia, inflammation, and the renin-angiotensin system^{272,279,280,387}, we should consider whether intracellular acidification elicited by glucose metabolism in islet cells could trigger a similar release of succinate, instigating SUCNR1 activation in β -cells.

To delve into this matter, it would be rational to explore the differential expression of dicarboxylate transporters and the application of assays that introduce isotope-labeled succinate into either the culture media or β -cell or islets themselves. These approaches would provide valuable insights into the activity of these transporters and allow for the quantification of succinate uptake and export under varying conditions, such as different glucose concentrations. Ultimately, such research could illuminate the intricate effects of extracellular succinate's autocrine and, if studied within islets, paracrine impacts on insulin secretion, enriching our comprehension of succinate's role on β -cell function.

Additionally, a critical element deserving comprehensive investigation pertains to the half-life of succinate in circulation. The modulation of circulating succinate levels is contingent upon an array of interconnected processes. These include succinate clearance, which encompasses mechanisms such as excretion⁵¹¹, cellular scavenging –as highlighted in Peruzzotti-Jametti et al.'s work on neuronal stem cells³⁹⁸–, and uptake for utilization, a topic explored by Mills et al. in the context of BAT thermogenesis³³⁶. An additional key determinant of circulating succinate levels is its endogenous generation, a process chiefly situated within the TCA cycle and subsequent

exportation. Notably, the role of the gut and its associated microbiota as substantial contributors to these processes cannot be understated and thus merits careful consideration. The complexity of these interdependent mechanisms, which might impact the endocrine role of succinate on insulin secretion, underscores the necessity for additional research to elucidate their precise functions and relationships.

Taken together, our findings lay the groundwork for exciting therapeutic opportunities while also indicating the need for a nuanced approach to leveraging the succinate-SUCNR1 axis. Balancing the multiple roles of this axis in different physiological contexts will be vital in devising effective treatments for a range of conditions, from metabolic disorders to inflammatory diseases.

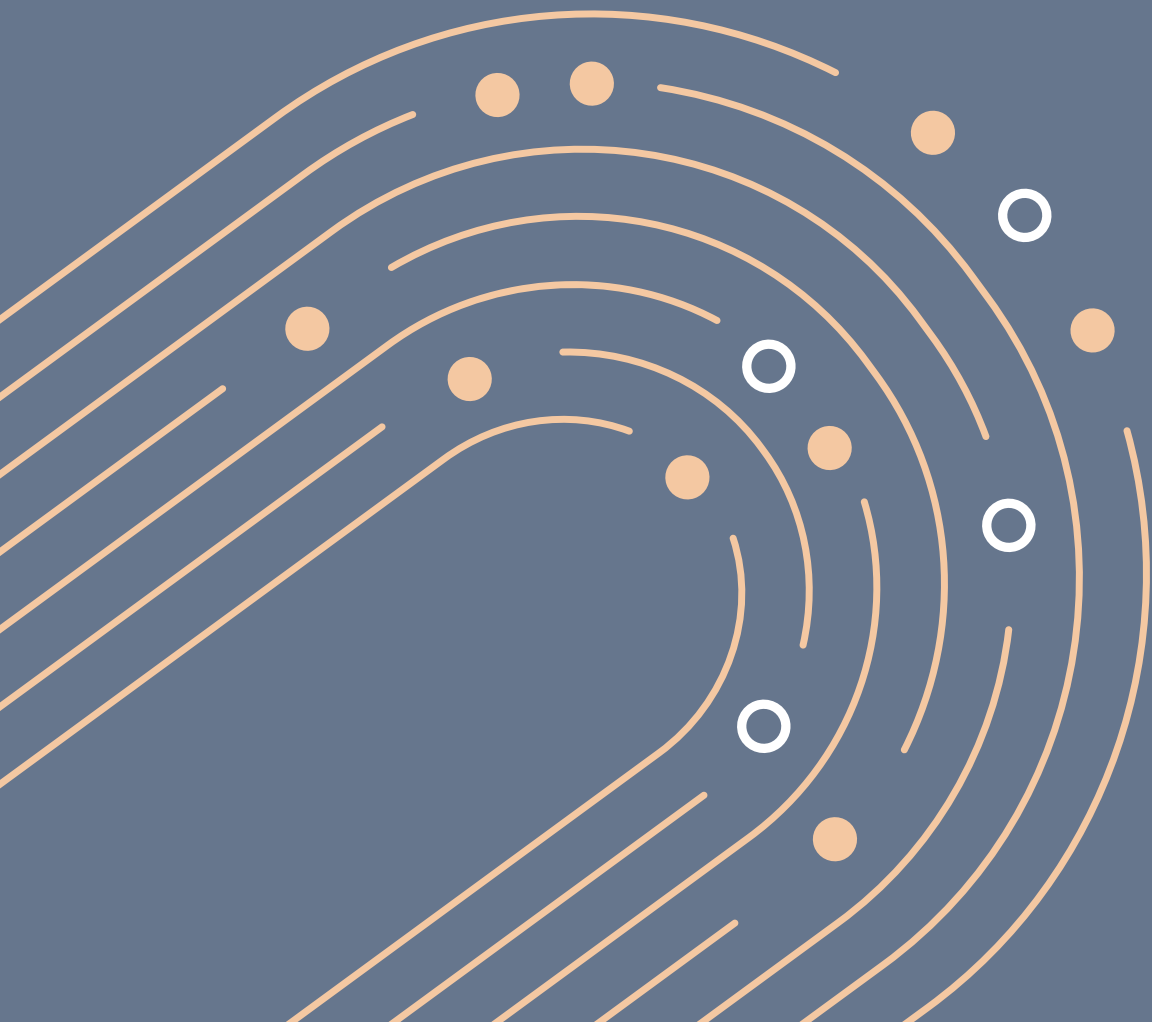
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UNCOVERING NOVEL INSIGHTS INTO INSULIN SECRETION REGULATION AND DIABETES PATHOGENESIS

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Conclusions

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1. SUCNR1, within the pancreas, exhibits a distinctive localization in islets, predominantly in β -cells.
2. Obesity and T2D are associated with a higher expression of SUCNR1 in pancreatic islets.
3. High glucose presence stimulates succinate release and SUCNR1 expression in β -cells.
4. Extracellular succinate, functioning through a SUCNR1-Gq-PKC mechanism, significantly enhances insulin secretion in hyperglycemia.
5. SUCNR1's role in β -cells proves essential for sustaining *in vivo* insulin secretion and glucose homeostasis under diet-induced insulin resistance conditions.
6. The increase in circulating succinate that is noted post-glucose administration, especially pronounced in individuals with prediabetes, combined with its significant correlation with insulin secretion, indicates that succinate might function as a hormone, facilitating the compensatory hyperinsulinemic response characteristic of prediabetic states.

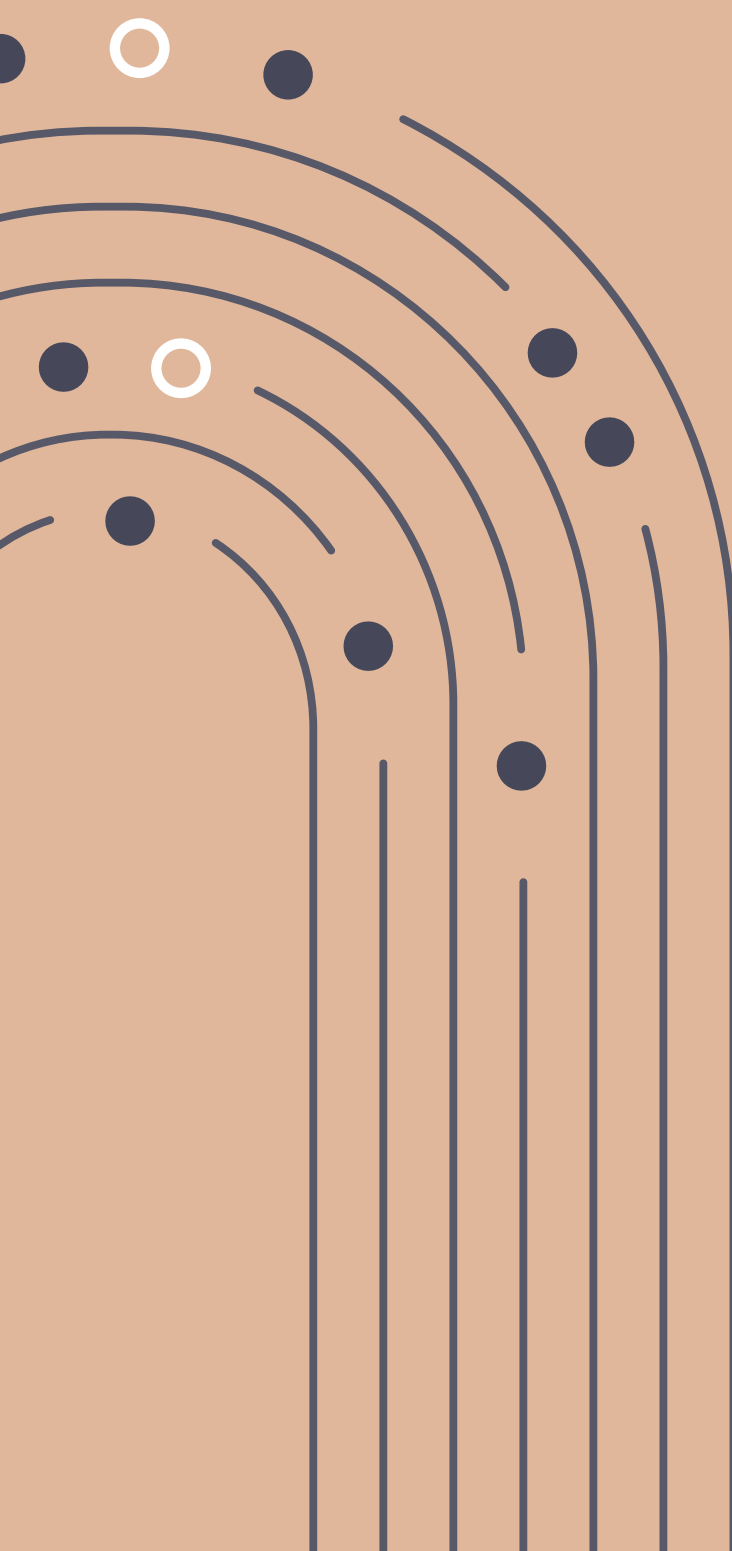
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References

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UNIVERSITAT ROVIRA I VIRGILI

THE SUCCINATE-SUCNR1 AXIS AND ITS IMPACT ON BETA-CELL FUNCTION: AN INTEGRATIVE STUDY
UNCOVERING NOVEL INSIGHTS INTO INSULIN SECRETION REGULATION AND DIABETES PATHOGENESIS

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Annex

8

UNIVERSITAT ROVIRA I VIRGILI

THE SUCCINATE-SUCNR1 AXIS AND ITS IMPACT ON BETA-CELL FUNCTION: AN INTEGRATIVE STUDY
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Joan-Martí Sabadell i Basallote

Supplementary Methods Information

Table 5. List of Employed Reagents and Resources in the Study

Reagent or Resource	Source	Database Identifier
Antibodies		
Anti-SUCNR1/GPR91, rabbit polyclonal	Novus Biologicals (Bio-Techne)	Cat.# NBP1-00861, RRID: AB_1503315
Anti-SUCNR1/GPR91, rabbit polyclonal	Novus Biologicals (Bio-Techne)	Cat.# NLS3476; RRID: AB_523816
Anti-β-Actin, mouse monoclonal	Sigma-Aldrich (Merck KGaA)	Cat.# A1978; RRID: AB_476692
Anti-Phospho-PKCδ (Thr505), rabbit polyclonal	Cell Signaling Technology	Cat.# 9374; RRID: AB_2168837
Anti-Phospho-PKCζ/λ (Thr410/403), rabbit polyclonal	Cell Signaling Technology	Cat.# 9378; RRID: AB_216821
Anti-Phospho-Akt (Ser473), rabbit monoclonal	Cell Signaling Technology	Cat.# 4058; RRID: AB_331168
Anti-Phospho-GSK3α/β (Ser21/9), rabbit polyclonal	Cell Signaling Technology	Cat.# 9331; RRID: AB_329830
Anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), rabbit polyclonal	Cell Signaling Technology	Cat.# 9101; RRID: AB_331646
Anti-Phospho-p38 MAPK (Thr180/Tyr182), rabbit monoclonal	Cell Signaling Technology	Cat.# 4511; RRID: AB_2139682
Anti-Phospho-CREB (Ser133), rabbit monoclonal	Cell Signaling Technology	Cat.# 9198; RRID: AB_2561044
Amersham ECL anti-rabbit IgG, horseradish peroxidase-linked species-specific whole antibody	Cytiva (Danaher Corporation)	Cat.# NA934V; Lot:#17572641
Anti-Insulin, mouse monoclonal	Sigma-Aldrich (Merck KGaA)	Cat.# I2018; RRID: AB_260137
Anti-Insulin, mouse monoclonal	Santa Cruz Biotechnology	Cat.# sc-8033; RRID: AB_627285
Anti-Glucagon, mouse monoclonal	Sigma-Aldrich (Merck KGaA)	Cat.# SAB4501137; RRID: AB_10761583
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 488	Invitrogen (Thermo Fisher Scientific)	Cat.# A32723; RRID: AB_2633275
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	Invitrogen (Thermo Fisher Scientific)	Cat.# A11031; RRID: AB_144696
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Invitrogen (Thermo Fisher Scientific)	Cat.# A-11029; RRID: AB_2534088
Goat anti-Mouse IgM (Heavy chain) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	Invitrogen (Thermo Fisher Scientific)	Cat.# A-21043; RRID: AB_2535712
Anti-Chromogranin A, mouse monoclonal	Dako (Agilent Technologies)	Cat.# M0869; RRID: AB_2081135
Human Biological Samples		
Human islets (for the mRNA expression analysis)	Hospital Universitari de Bellvitge and IDIBELL	N/A
Human islets (for the protein abundance analysis)	Integrated Islet Distribution Program and Prodo Laboratories	Listed in Table 2 (Materials and Methods chapter, pg. 77)
Chemicals, Peptides, and Recombinant Proteins		
Sodium chloride (NaCl)	Panreac Química SLU (Illinois Tool Works Inc.)	Cat.# 131659.1211; CAS ID: 7647-14-5

Potassium chloride (KCl)	Panreac Química SLU (Illinois Tool Works Inc.)	Cat.# 131494; CAS ID: 7440-09-7
Calcium chloride (CaCl ₂)	Thermo Fisher Scientific	Cat.# c/1500/53; CAS ID: 10043-52-4
Magnesium chloride (MgCl ₂)	Sigma-Aldrich (Merck KGaA)	Cat.# M8266; CAS ID: 7786-30-3
Sodium bicarbonate (NaHCO ₃)	Merck KGaA	Cat.# 6329; CAS ID: 144-55-8
Sodium phosphate monobasic (NaH ₂ PO ₄)	Sigma-Aldrich (Merck KGaA)	Cat.# S3139; CAS ID: 7558-80-7
Sodium phosphate dibasic (Na ₂ HPO ₄)	Sigma-Aldrich (Merck KGaA)	Cat.# S9763; CAS ID: 7558-79-4
D-(+)-Glucose (C ₆ H ₁₂ O ₆)	Sigma-Aldrich (Merck KGaA)	Cat.# G8270; CAS ID: 50-99-7
Fibronectin bovine plasma	Sigma-Aldrich (Merck KGaA)	Cat.# F1141; CAS ID: 86088-83-7
Human serum albumin 25%	Grifols Inc.	N/A
Bovine serum albumin fraction V, fatty acid free	Roche	Cat.# 10775835001; CAS ID: 9048-46-8
Nicotinamide (C ₆ H ₆ N ₂ O)	Sigma-Aldrich (Merck KGaA)	Cat.# N3376; CAS ID: 98-92-0
Human transferrin	Sigma-Aldrich (Merck KGaA)	Cat.# T8158; CAS ID: 11096-37-0
Sodium selenite (Na ₂ SeO ₃)	Sigma-Aldrich (Merck KGaA)	Cat.# 214485; CAS ID: 10102-18-8
Penicillin-Streptomycin (10,000 U/mL)	Gibco (Thermo Fisher Scientific)	Cat.# 15140122
GlutaMAX™ Supplement (100X)	Gibco (Thermo Fisher Scientific)	Cat.# 35050061
Trypsin from porcine pancreas	Sigma-Aldrich (Merck KGaA)	Cat.# T4799; CAS ID: 9002-07-7
DNase I, recombinant, RNase-free	Roche	Cat.# 04716728001; CAS ID: 9003-98-9
Propidium iodide	Sigma-Aldrich (Merck KGaA)	Cat.# P4170; CAS ID: 25535-16-4
Hoechst 33342	Sigma-Aldrich (Merck KGaA)	Cat.# B2261; CAS ID: 875756-97-1
4',6-Diamidino-2-phenylindole dihydrochloride, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI)	Sigma-Aldrich (Merck KGaA)	Cat.# D9542; CAS ID: 28718-90-3
3-isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich (Merck KGaA)	Cat.# I5879; CAS ID: 28822-58-4
Actrapid® Insulin, human recombinant (100 IU/ml)	Novo Nordisk	ATC.#A10AB01
2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid (HEPES)	Thermo Fisher Scientific	Cat.# BP310-1; CAS ID: 7365-45-9
Cis-epoxysuccinic acid (cESA)	TCI Chemicals	Cat.# E0449; CAS ID: 16533-72-5
Disodium succinate	Sigma-Aldrich (Merck KGaA)	Cat.# W327700; CAS ID: 150-90-3
NF-56-EJ40	MedChemExpress	Cat.# HY-130246; CAS ID: 2380230-73-7
Gö 6983	Tocris (Bio-Techne)	Cat.# 2285; CAS ID: 133053-19-7

FR900359	Cayman Chemical Co.	Cat.# 33666; CAS ID: 107530-18-7
Formaldehyde solution 4%, buffered, pH 6.9	Sigma-Aldrich (Merck KGaA)	Cat.# 1004965000
Xylene (isomeric mixture)	EMD Millipore (Merck KGaA)	Cat.# 108298; CAS ID: 1330-20-7
Tris base, Trizma® base	Sigma-Aldrich (Merck KGaA)	Cat.# 10708976001; CAS ID: 77-86-1
Tris hydrochloride	Thermo Fisher Scientific	Cat.# BP153-500; CAS ID: 1185-53-1
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (Merck KGaA)	Cat.# E6758; CAS ID: 60-00-4
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich (Merck KGaA)	Cat.# L3771; CAS ID: 151-21-3
40% Acrylamide/Bis Solution	Bio-Rad	Cat.# 1610144
Ammonium persulfate $[(\text{NH}_4)_2\text{S}_2\text{O}_8]$	Sigma-Aldrich (Merck KGaA)	Cat.# A3678; CAS ID: 7727-54-0
N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)	Thermo Fisher Scientific	Cat.# 17919; CAS ID: 110-18-9
Blotting Grade Blocker Non Fat Dry Milk	Bio-Rad	Cat.# 1706404XTU
Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)	Sigma-Aldrich (Merck KGaA)	Cat.# E3889; CAS ID: 67-42-5
2-mercaptoethanol	Sigma-Aldrich (Merck KGaA)	Cat.# M3148; CAS ID: 60-24-2
TRIzol™ Reagent	Invitrogen (Thermo Fisher Scientific)	Cat.# 15596018
Harris Hematoxylin	StatLab Medical Products	Cat.# HXHHELT; CAS ID: 517-28-2
Eosin	Sigma-Aldrich (Merck KGaA)	Cat.# HT110216
TWEEN® 20	Sigma-Aldrich (Merck KGaA)	Cat.# P2287; CAS ID: 9005-64-5
Fura-2 acetoxymethyl ester, cell permeant	Invitrogen (Thermo Fisher Scientific)	Cat.# F1221; CAS ID: 108964-32-5
SYBR Safe DNA-binding dye	Thermo Fisher Scientific	Cat.# S33102; CAS ID: 1030826-36-8
Collagenase NB 1 Premium Grade	Serva Electrophoresis GmbH	Cat.# DS17455.03; CAS ID: 9001-12-1
Neutral Protease NB GMP Grade	Serva Electrophoresis GmbH	Cat.# DS30303.01; CAS ID: 9001-92-7
Gentamycin, Genta-Gobens	Laboratorios Normon	Cat.# 882811.1; CAS ID: 1403-66-3
Amphotericin B	Gibco (Thermo Fisher Scientific)	Cat.# 15290018; CAS 1397-89-3
Ciprofloxacin	Fresenius Kabi AG	CAS 85721-33-1
Commercial Assays		
EnzyChrom™ Succinate Assay Kit	BioAssay Systems	Cat.# ESNT-100
Multi Species GLP-1 Total ELISA	EMD Millipore (Merck KGaA)	Cat.# EZGLP1T-36K
Human Insulin ELISA kit	Mercodia	Cat.# 10-1113-01

Ultrasensitive Mouse Insulin ELISA kit	Mercodia	Cat.# 10-1249-01
Mouse GLP-1 ELISA kit	Crystal Chem	Cat.# 81508
EnVision FLEX DAB+ Substrate Chromogen System	Dako (Agilent Technologies)	Cat.# GV82511-2
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	Cat.# 23225
Pierce™ ECL Western Blotting Substrate	Thermo Fisher Scientific	Cat.# 32106
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Fisher Scientific	Cat.# 34095
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor	Applied Biosystems (Thermo Fisher Scientific)	Cat.# 4374966
TaqMan™ Universal PCR Master Mix	Applied Biosystems (Thermo Fisher Scientific)	Cat.# 4304437
DreamTaq Green DNA Polymerase Kit	Thermo Fisher Scientific	Cat.# K1081
Other Commercial Resources		
100 bp DNA ladder	Thermo Fisher Scientific	Cat.# 15628050
PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	Thermo Fisher Scientific	Cat.# 26619
Lysis Solution for Blood	Sigma-Aldrich (Merck KGaA)	Cat.# L3289
Neutralization Solution for Blood	Sigma-Aldrich (Merck KGaA)	Cat.# N9784
Normal-control diet, 3.1 kcal% fat	SAFE Diets	Cat.# A04
High-fat diet, 60 kcal% fat	Research Diets, Inc.	Cat.# D12492
Miami Media #1A Culture Medium (with Human Serum Albumin, without Antibiotics)	Corning Inc.	Cat.# 98-021-CV
Ham's F-10 Nutrient Mix	Gibco (Thermo Fisher Scientific)	Cat.# 11550043
HyClone™ Dulbecco's Modified Eagle Medium with high glucose	Cytiva (Danaher Corporation)	Cat.# SH30081.01
HyClone™ Dulbecco's phosphate buffered saline (DPBS), without calcium and magnesium	Cytiva (Danaher Corporation)	Cat.# SH30028.02
Dulbecco's Modified Eagle's Medium, 1 g/l glucose	Corning Inc.	Cat.# 10-014-CV
Dulbecco's Modified Eagle Medium, no glucose	Gibco (Thermo Fisher Scientific)	Cat.# 11-966-025
CMRL1066 medium	Corning Inc.	Cat.# 15-110-CV
HyClone™ Fetal Bovine Serum (FBS)	Cytiva (Danaher Corporation)	Cat.# SH30071.03
Donkey serum	Sigma-Aldrich (Merck KGaA)	Cat.# D9663
ECM Gel from Engelbreth-Holm-Swarm murine sarcoma	Sigma-Aldrich (Merck KGaA)	Cat.# E1270

βCOAT matrix	Human Cell Design	N/A
ULTI-β1 media	Human Cell Design	N/A
ULTI-ST media	Human Cell Design	N/A
βKrebs media	Human Cell Design	N/A
Immobilon-P PVDF membrane	EMD Millipore (Merck KGaA)	Cat.# IPVH00010
Mammalian Protein Extraction Reagent (M-PER™)	Thermo Fisher Scientific	Cat.# 78501
Halt™ Phosphatase Inhibitor Cocktail 100X	Thermo Fisher Scientific	Cat.# 78426
Halt™ Protease Inhibitor Cocktail 100X	Thermo Fisher Scientific	Cat.# 78429
Citrate Buffer (pH 6.0), Concentrate	Thermo Fisher Scientific	Cat.# 005000
10X Tris/Glycine/SDS	Bio-Rad	Cat.# 1610732
Experimental Models: Cell Lines		
MIN6 cell line	Provided by Dr. Rosa Gasa (IDIBAPS, Barcelona)	RRID: CVCL_0431
EndoC-βH1 cell line	Human Cell Design	RRID: CVCL_L909
EndoC-βH5 cell line	Human Cell Design	RRID: CVCL_C7TU
Experimental Models: Organisms and Strains		
Wistar rats	Charles River Laboratories	N/A
Wild type C57BL/6 mice	Charles River Laboratories	N/A
BKS.Cg-Dock7 ^m +/+Lepr ^{db} J mice (db/db mice)	Charles River Laboratories	N/A
B6(Cg)-Ins1 ^{tm1.1(cre)Thor} /J mice (Ins1 ^{Cre} mice)	Dr. Jorge Ferrer ⁴³⁶	N/A
Sucnr1 ^{fl/fl} mice	Obtained from our previous work ³⁹⁵	N/A
Oligonucleotides		
Mouse Ins1 common genotyping forward primer: 5'-GGCCAAACAGCAAAGTCCAG-3'	Integrated DNA Technologies	N/A
Mouse Ins1 wild-type allele reverse primer: 5'-GATCCACAATGCCACGCTTC-3'	Integrated DNA Technologies	N/A
Mouse Ins1 Cre knockin allele reverse primer: 5'-AACCAGCGTTTTTCGTTCTGC-3'	Integrated DNA Technologies	N/A
Mouse Sucnr1 genotyping forward primer: 5'-AGACAAGTTATGACTATATGCTGAGAC-3'	Integrated DNA Technologies	N/A
Mouse Sucnr1 genotyping reverse primer: 5'-TTCACATCTATAATATAGCACCTGTA-3'	Integrated DNA Technologies	N/A
TaqMan™ human SUCNR1 probe	Applied Biosystems (Thermo Fisher Scientific)	Cat.# Hs00908230_m1
TaqMan™ human GLP1R probe	Applied Biosystems (Thermo Fisher Scientific)	Cat.# Hs00157705_m1

TaqMan™ human <i>TBP</i> probe	Applied Biosystems (Thermo Fisher Scientific)	Cat.# Hs00427620_m1
TaqMan™ rat <i>Sucnr1</i> probe	Applied Biosystems (Thermo Fisher Scientific)	Cat.# Rn02084929_s1
TaqMan™ rat <i>B2m</i> probe	Applied Biosystems (Thermo Fisher Scientific)	Cat.# Rn00560865_m1
TaqMan™ mouse <i>Sucnr1</i> probe	Applied Biosystems (Thermo Fisher Scientific)	Cat.# Mm02620543_m1
TaqMan™ mouse <i>B2m</i> probe	Applied Biosystems (Thermo Fisher Scientific)	Cat.# Mm00437762_m1
TaqMan™ mouse <i>18s</i> probe	Applied Biosystems (Thermo Fisher Scientific)	Cat.# Mm04277571_s1
Software and Algorithms		
Fiji software v2.9.0	National Institutes of Health, USA	RRID: SCR_002285
iBright analysis software	Invitrogen (Thermo Fisher Scientific)	RRID: SCR_017632
Pannoramic 250 Flash III slide scanner, SlideViewer v2.6 software	3DHISTECH Ltd.	RRID: SCR_022184
FL WinLab v2.0	PerkinElmer	N/A
Islet Regulome Browser ⁴⁴¹	http://www.isletregulome.org	N/A
GraphPad Prism 9	GraphPad Software	RRID: SCR_002798
IBM SPSS Statistics v27.0	IBM Corp	RRID: SCR_002865
Custom software generated by Andrea Tura and Andrea Mari ²¹³ based on MATLAB	MathWorks, Inc.	RRID: SCR_001622
ZEN Digital Imaging for Light Microscopy v3.1 (blue edition)	Zeiss	RRID: SCR_013672
BioRender.com	BioRender	RRID: SCR_018361

CAS ID: Chemical Abstracts Service Identifier; Cat.#: catalog number; RRID: Research Resource Identifier.

Scientific Publications During the Doctoral Studies

PUBLISHED ARTICLES

Title: SUCNR1 signaling in adipocytes controls energy metabolism by modulating circadian clock and leptin expression.

Authors: Teresa Villanueva-Carmona, Lúdia Cedó, Ana Madeira, Victòria Ceperuelo-Mallafré, M-Mar Rodríguez-Peña, Catalina Núñez-Roa, Elsa Maymó-Masip, Maria Repollés-de-Dalmau, Joan Badia, Noelia Keiran, Mercedes Mirasierra, Carolina Pimenta-Lopes, **Joan Sabadell-Basallote**, Ramón Bosch, Laura Caubet, Joan Carles Escolà-Gil, José-Manuel Fernández-Real, Nuria Vilarrasa, Francesc Ventura, Mario Vallejo, Joan Vendrell, Sonia Fernández-Veledo.

Position: 13/22

Journal: Cell Metabolism

Journal metrics:

- **Impact factor:** 31.373
- **Rank by discipline:** 3/146 Endocrinology & Metabolism (D1), 8/195 Cell Biology (D1)

Citation: Villanueva-Carmona T, Cedó L, Madeira A, et al. SUCNR1 signaling in adipocytes controls energy metabolism by modulating circadian clock and leptin expression. *Cell Metab.* 2023;35(4):601-619.e10. doi:10.1016/j.cmet.2023.03.004.

Title: Glycogen accumulation in adipocyte precursors from elderly and obese subjects triggers inflammation via SIRT1/6 signaling.

Authors: Margarida Terrón-Puig, Isabel Huber-Ruano, **Joan Sabadell-Basallote**, Miriam Ejarque, Catalina Núñez-Roa, Elsa Maymó-Masip, Rosa Jorba, Carolina Serena, Joan Vendrell, Sonia Fernández-Veledo.

Position: 3/10

Journal: Aging Cell

Journal metrics:

- **Impact factor:** 11.005
- **Rank by discipline:** 4/51 Geriatrics & Gerontology (D1), 30/195 Cell Biology (Q1)

Citation: Terrón-Puig M, Huber-Ruano I, Sabadell-Basallote J, et al. Glycogen accumulation in adipocyte precursors from elderly and obese subjects triggers inflammation via SIRT1/6 signaling. *Aging Cell*. 2022;21(8):e13667. doi:10.1111/accel.13667.

Title: Diabetes alters the protein secretome of human adipose-derived stem cells and promotes tumorigenesis in hepatic cancer cells.

Authors: Miriam Ejarque*, **Joan Sabadell-Basallote***, Ester Benaiges, Catalina Núñez-Roa, Eduardo Sabido, Eva Borrás, Erik Llacer, Antonio Zorzano, Joan Vendrell, Sonia Fernández-Veledo.

Position: 1/10 (*co-first)

JOURNAL: Clinical and Translational Medicine

Journal metrics:

- **Impact factor:** 8.554
- **Rank by discipline:** 22/195 Medicine, Research & Experimental (Q1), 37/318 Oncology (Q1)

Citation: Ejarque M, Sabadell-Basallote J, Benaiges E, et al. Diabetes alters the protein secretome of human adipose-derived stem cells and promotes tumorigenesis in hepatic cancer cells. *Clin Transl Med*. 2022;12(6):e823. doi:10.1002/ctm2.823.

Title: Adipose tissue is a key organ for the beneficial effects of GLP-2 metabolic function.

Authors: Miriam Ejarque, **Joan Sabadell-Basallote**, Daniel Beiroa, Enrique Calvo, Noelia Keiran, Catalina Núñez-Roa, María del Mar Rodríguez, Fati-

ma Sabench, Daniel del Castillo, Veronica Jimenez, Fatima Bosch, Ruben Nogueiras, Joan Vendrell, Sonia Fernández-Veledo.

Position: 2/14

Journal: British Journal of Pharmacology

Journal metrics:

- **Impact factor:** 9.473
- **Rank by discipline:** 18/279 Pharmacology & Pharmacy (D1)

Citation: Ejarque M, Sabadell-Basallote J, Beiroa D, et al. Adipose tissue is a key organ for the beneficial effects of GLP-2 metabolic function. *Br J Pharmacol.* 2021;178(10):2131-2145. doi:10.1111/bph.15278.

Title: Effects of stem cells from inducible brown adipose tissue on diet-induced obesity in mice.

Authors: Enrique Calvo, Noelia Keiran, Catalina Núñez-Roa, Elsa Maymó-Masip, Miriam Ejarque, **Joan Sabadell-Basallote**, María del Mar Rodríguez-Peña, Victòria Ceperuelo-Mallafre, Jesús Seco, Ester Benaiges, Theodora Michalopoulou, Rosa Jorba, Joan Vendrell, Sonia Fernández-Veledo.

Position: 6/14

Journal: Scientific Reports

Journal metrics:

- **Impact factor:** 4.997
- **Rank by discipline:** 19/135 Multidisciplinary Sciences (Q1)

CITATION: Calvo E, Keiran N, Núñez-Roa C, et al. Effects of stem cells from inducible brown adipose tissue on diet-induced obesity in mice. *Sci Rep.* 2021;11(1):13923. doi:10.1038/s41598-021-93224-6.

SUBMITTED MANUSCRIPTS

Title: The Succinate Receptor SUCNR1 Regulates Insulin Secretion with Implications in People with Prediabetes

Authors: Joan Sabadell-Basallote, Brenno Astiarraga, Miriam Ejarque, Maria Repollés-de-Dalmau, Jordi Blanco, Catalina Núñez-Roa, M-Mar Rodríguez-Peña, Laia Martínez, Dario F. De Jesus, Laura Marroquí, Ramon Bosch, Eduard Montanya, Francesc Sureda, Andrea Tura, Andrea Mari, Rohit N. Kulkarni, Joan Vendrell, Sonia Fernández-Veledo.

Position: 1/18

Journal: Journal of Clinical Investigation

Journal metrics:

- **Impact factor:** 19.477
- **Rank by discipline:** 2/139 Medicine, Research & Experimental (D1)

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Title: EndoC- β H5 cells are storable and ready-to-use human pancreatic beta cells with physiological insulin secretion.

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The Succinate Receptor SUCNR1 Regulates Insulin Secretion with Implications in People with Prediabetes

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ABSTRACT

Pancreatic β -cell dysfunction is a key feature of type 2 diabetes, and novel regulators of insulin secretion are desirable. Here we report that the succinate receptor (SUCNR1) is expressed in β -cells and is up-regulated in hyperglycemic states in mice and humans. We found that succinate acts as a hormone-like metabolite and stimulates insulin secretion via a SUCNR1-Gq-PKC-dependent mechanism in human β -cells. Mice with β -cell-specific *Sucnr1* deficiency exhibit impaired glucose tolerance and insulin secretion on a high-fat diet, indicating that SUCNR1 is essential for preserving insulin secretion in diet-induced insulin-resistance. Patients with impaired glucose tolerance show an enhanced nutritional-related succinate response, which correlates with the potentiation of insulin secretion during intravenous glucose administration. These data demonstrate that the succinate/SUCNR1 axis is activated by high glucose and identifies a novel GPCR-mediated amplifying pathway for insulin secretion relevant to the hyperinsulinemia of prediabetic states.

INTRODUCTION

Insulin secretion by pancreatic β -cells is crucial for blood glucose homeostasis and is tightly controlled by a complex network of hormones, nutrients, and neurotransmitters. Impaired β -cell function worsened by the compensatory hyperinsulinemia associated with insulin-resistant states is a key contributing factor of type 2 diabetes (T2D), a major chronic health concern primarily linked to obesity (1–4). Two different pathways are known to interact in β -cells to ensure insulin secretion in response to glucose stimulation. The well-known “triggering” pathway involves the mitochondrial metabolism of glucose, which induces the closure of ATP-sensitive potassium channels and the activation of voltage-gated Ca^{2+} channels, ultimately triggering the exocytosis of insulin granules (5). However, a second or “metabolic amplifying” pathway is necessary for proper insulin secretion response, involving the external replenishment of the Krebs or tricarboxylic acid (TCA) cycle via anaplerosis (6), and is distinct to the neurohormonal amplification pathways (e.g., incretin system) that are related to the activation of G protein-coupled receptors (GPCRs) (7). In addition to this dual and hierarchical control of insulin secretion, emerging evidence points to important roles for anaplerotic TCA cycle substrates that act as second messengers in the cytosol to drive insulin granule exocytosis (8). Within this framework, energy metabolites are increasingly recognized as signaling molecules (9).

In recent years, the TCA cycle substrate succinate has become the poster child for the concept that metabolites can have functions beyond energy metabolism. Cytosolic succinate accumulation occurs largely in response to aerobic glycolysis, breakdown of the TCA cycle and anaplerosis (10). Succinate can also be released into the extracellular space by specific plasma membrane transporters (11, 12), where it behaves akin to classical hormones and cytokines through engagement with succinate receptor 1 (SUCNR1) (10). Additionally, it has long been known that cell-permeable succinate esters are approximately one-third as potent as glucose at stimulating insulin release, and it is hypothesized that the insulinotropic properties of succinate are based on its capacity to increase the production of mevalonate and NADPH (13–16). Succinate might also act as a stimulus-coupling secondary signal for proinsulin biosynthesis independently of insulin secretion (17, 18). Importantly, succinate has been described as the TCA cycle metabolite more closely associated with second-phase insulin secretion (19). Nevertheless, the extramitochondrial and extracytosolic roles of succinate in insulin secretion are unexplored.

SUCNR1 expression has been observed in the pancreas (20, 21) and in human islets (22), indicating a potential instructive role for the succinate/SUCNR1 axis in islet physiology. Elevated circulating levels of succinate and SUCNR1 activation have been traditionally ascribed to hypoxia, tissue damage, and inflammatory processes (23). Correspondingly, SUCNR1 signaling has been primarily linked to pathophysiology, including metabolic disorders such as obesity, diabetes, and non-alcoholic fatty liver disease (24–31). Nonetheless, previous research has also described a transient rise in extracellular succinate during physiological processes, including exercise (12, 32), with which it is involved in muscle innervation and matrix remodeling *via* SUCNR1 (12). Also, we recently demonstrated that dynamic succinate regulation occurs during fasted-to-fed transition, which is potentiated when nutrients are sensed by the intestine but is disturbed in unhealthy metabolic states (33). Therefore, the initial view of SUCNR1 as an exclusive sensor for local damage is being reshaped in the light of recent observations of succinate as a systemic metabolic signal.

SUCNR1 can signal through cAMP- or Ca^{2+} -dependent pathways (34–36), which are well-known regulators of insulin secretion in β -cells (37). Notably, global *Sucnr1*-knockout mice fed high-fat diet (HFD) show defective insulin secretion and glucose intolerance, without significant changes in β -cell mass (25), raising suspicion of its role as a regulator of β -cell function. Here, we adopted a comprehensive and integrative translational approach to investigate the impact of the succinate/SUCNR1 axis on the β -cell function. Our investigation using a unique pancreatic

β -cell-specific knockout mouse model and *in vitro* analyses demonstrate that the succinate/SUCNR1 axis serves a glucose-dependent, novel amplifying transduction pathway enhancing insulin release. Furthermore, we find that succinate functions as an incretin-like metabolite, which significantly potentiates insulin secretion in hyperglycemia in humans.

RESULTS

Pancreatic β -cells express SUCNR1

We first analyzed *Sucnr1* mRNA levels in metabolic tissues from mice (**Figure 1A**), confirming *Sucnr1* expression in the total pancreas, albeit at low levels compared with white adipose tissue and liver, in agreement with previous reports (20, 21, 38). Immunohistochemical staining of pancreas sections revealed a higher SUCNR1 protein abundance in islets than in exocrine tissue in both human and mouse samples (**Figure 1B**). To gain insight into the cell type-specific expression of SUCNR1 in pancreatic islets, we examined its mRNA levels in the two major cell populations: α - and β -cells (39). Results showed that *Sucnr1* was specifically expressed in β -cells from rat islets (**Figure 1C**). Gene expression regulation differs across tissues, and particular factors may influence *SUCNR1* expression in human islets. *In silico* analysis of the human *SUCNR1* gene using the Islet Regulome Browser (40) identified NKX2.2, FOXA2, and NKX6.1 as putative regulators of *SUCNR1* expression, which are key transcription factors for β -cell identity and function (41–43). Bioinformatics analysis also revealed *SUCNR1* expression in a published RNA-seq library from human islets (44). Finally, the *SUCNR1* locus and its surrounding regions contained several single-nucleotide polymorphisms identified in patients with T2D in a genome-wide association study dataset (45) (**Figure 1D**).

SUCNR1 expression in islets is regulated in human obesity and T2D

To evaluate whether *Sucnr1* is regulated in the pancreas in different metabolic states, we first assessed its expression in total pancreas tissue from rodent models of obesity and T2D. We observed a trend for elevated expression in diet-induced obese (DIO) mice and a further trend increase in expression in diabetic (*db/db*) mice (**Figure 2A**). Comparable but significant results were obtained in human pancreatic islets from patients with obesity/T2D in two independent cohorts, without signifi-

cant changes in the levels of *GLP1R* (**Figure 2B**, donor information in **Supplemental Table 1**). These results were confirmed at the protein level in human islet lysates (**Figure 2C**, donor information in **Supplemental Table 2**). In addition, we found a positive correlation between *SUCNR1* mRNA/protein levels in islets and the body mass index (BMI) of the human donors (**Figure 2D**). Overall, these data indicate that islet *SUCNR1* is associated with changes in metabolic status, implying a potential role of this receptor in the pathophysiology of metabolic disorders and T2D.

β -cells sense extracellular succinate and release insulin in a *SUCNR1*-Gq-PKC-dependent mechanism

We next explored a potential link between hyperglycemia and the succinate/*SUCNR1* axis in β -cells. Glucose catabolism increases the intracellular levels of succinate in β -cells through pyruvate oxidation *via* the TCA cycle (46). We observed that high-glucose exposure of mouse-derived β -cell line MIN6 stimulated succinate release to the extracellular medium (**Figure 3A**). As pancreatic β -cells can regulate their behavior in response to glucose, we analyzed *Sucnr1* expression in MIN6 cells cultured at different glucose concentrations, finding that glucose increased *Sucnr1* expression in a dose-dependent manner (**Figure 3B**). This result was confirmed at the protein level in the human β -cell line EndoC- β H1 cultured at increasing glucose concentrations (**Figure 3C**).

Our findings that β -cells might increase their sensitivity to extracellular succinate by increasing *SUCNR1* expression in response to glucose in a dose-dependent manner prompted us to investigate whether *SUCNR1* activation impacts insulin secretion. We observed that extracellular succinate significantly potentiated (20% increase) glucose-stimulated insulin secretion (GSIS) in MIN6 cells (**Figure 3D**). Even though succinate in culture medium takes an anionic form, and as such is widely considered as non-cell-permeable (16), we also utilized the synthetic *SUCNR1* agonist *cis*-epoxysuccinic acid (cESA), which has a lower EC_{50} compared to succinate (47), to exclude any intracellular effects of succinate induced by its uptake into cells *via* dicarboxylic acid transporters (48, 49). Results showed that cESA similarly augmented insulin secretion under high glucose (**Figure 3D**).

The effect of succinate as an extracellular insulin secretagogue was confirmed in EndoC- β H5 cells which, compared with other commonly used human β -cell lines, do not present limitations regarding the expression of some GPCRs (i.e., *GLP1-R*) (50). EndoC- β H5 cells showed an increase in insulin secretion when *SUCNR1* was

activated by both extracellular succinate or cESA under conditions of no glucose (1.5-fold or 1.3-fold increase, respectively) and high-glucose (1.4-fold increase for both) conditions (**Figure 3E**). To further demonstrate that the effect of succinate on insulin release is dependent on its extracellular-driven signaling properties, we co-treated cells with the human-specific SUCNR1 antagonist NF-56-EJ40 (51) during the GSIS assay. Acute receptor antagonism completely blocked succinate-induced insulin secretion in the presence of high-glucose (**Figure 3F**), confirming that the insulintropic effect of extracellular succinate is dependent on SUCNR1.

We next investigated the underlying mechanism of insulin release by SUCNR1 in β -cells. While SUCNR1 transduction pathways remain largely elusive (52), it is increasingly recognized that they are cell- and context-dependent. We thus challenged MIN6 cells with succinate or cESA and analyzed several canonical proteins that might integrate GPCR signal transduction. Results of western blotting revealed a succinate-dependent increase in the phosphorylated forms of AKT and the novel and atypical PKCs, PKC δ , and PKC ζ , respectively, which was accompanied by the differential phosphorylation of ERK1/2 and p38 MAPK (**Figure 4A**). Concomitant with this activation, there was an increase in phosphorylated CREB and phosphorylated (inactive) GSK-3 α/β . As β -cells are sensitive to changes in $[Ca^{2+}]_i$, we also assessed Ca^{2+} mobilization in MIN6 cells upon SUCNR1 activation using a fluorometric assay to measure real-time changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$). We first challenged low-glucose-cultured MIN6 cells with additive doses of succinate, observing no changes in $[Ca^{2+}]_i$. However, when glucose in the culture medium was increased to 16.7 mM, $[Ca^{2+}]_i$ was progressively mobilized with increasing additions of succinate (ANOVA after glucose addition $p=0.0126$). As cells were exposed to increasing succinate levels before high-glucose stimulation, we studied the succinate effect on Ca^{2+} mobilization immediately after glucose addition. Notably, we also observed a significant glucose-dependent succinate dose-response on $[Ca^{2+}]_i$ in MIN6 cells at lower extracellular succinate doses, which reached significance at 800 μ M succinate (ANOVA after glucose addition $p=0.0013$) (**Figure 4B**).

Within the framework of neurohormonal amplification pathways, PKC activation is almost universally accepted as a major mechanism of GPCR-stimulated insulin secretion (53, 54). To discern the contribution of this family of serine-threonine kinases to SUCNR1-induced insulin secretion, we treated EndoC- β H5 cells with the pan-PKC inhibitor Gö 6983, finding that it partially blocked insulin release stimulated by succinate in high-glucose conditions (**Figure 4C**). Several G proteins are known to mediate GPCR signaling upon receptor activation (55). Given our finding

that PKC signaling is implicated in SUCNR1-induced insulin secretion, we assessed the involvement of Gq, which is situated upstream of PKC (56). We used FR900359 to inhibit Gq activity in EndoC- β H5 cells, observing a complete blockade of succinate-induced potentiation of insulin release (**Figure 4D**). Overall, our data suggest the succinate/SUCNR1 axis is a novel regulator of insulin secretion in a Gq- and PKC-dependent manner.

Specific β -cell ablation of SUCNR1 induces glucose intolerance due to β -cell impairment

To further study the function of SUCNR1 in pancreatic β -cells *in vivo*, we generated mice with β -cell-specific *Sucnr1* deletion. We crossed mice carrying a conditional *Sucnr1* loxP-flanked locus (*Sucnr1^{fl/fl}*) with knock-in heterozygous transgenic mice in which the Cre recombinase gene is introduced at the initiator codon of the *Ins1* endogenous gene locus (*Ins1^{Cre/+}*). Accordingly, *Ins1^{Cre/+}* mice do not show recombination in the brain and have similar glucose homeostasis and body weight gains as wild-type mice (57). The resulting offspring included both control mice (*Ins1^{+/+} Sucnr1^{fl/fl}*, referred to as *Sucnr1^{fl/fl}* or control mice) and mice with β -cell-specific *Sucnr1* ablation (*Ins1^{Cre/+} Sucnr1^{fl/fl}*, referred to as *Sucnr1*- β KO mice) (**Supplemental Figure 1**). These mice were born in a typical Mendelian fashion with no recognizable morphological differences between genotypes (data not shown). Immunohistochemical staining revealed a pronounced decrease in SUCNR1 content within pancreatic islets of *Sucnr1*- β KO mice (**Figure 5A**). No differences between genotypes were evident on a normal control diet (NCD) in terms of body weight, glucose and insulin tolerance, and insulin secretion at 20 and 54 weeks of age (**Supplemental Figures 2 and 3**).

We next challenged control and *Sucnr1*- β KO mice to HFD to investigate whether a diet-induced metabolic stress phenotype existed. We found no differences in body weight between groups after 8 weeks of HFD feeding (**Figure 5B**). Examination of pancreatic islet morphology revealed a trend for lower β -cell mass in *Sucnr1*- β KO mice than in controls, without any evident change in β -cell mass (**Figure 5C**). Remarkably, *Sucnr1*- β KO mice showed an overall higher diet-induced hyperglycemia than control mice, which was particularly significant in random-fed conditions (**Figure 5D**). Simultaneously, *Sucnr1*- β KO mice showed lower plasma insulin levels, which was also notably significant in random-fed conditions (**Figure 5E**).

To question whether specific β -cell disruption of *Sucnr1* affected glucose response in an obesity context, we performed a glucose tolerance test (GTT) in mice fed HFD.

To consider potential differences in incretin-induced responses in β -cells, we independently challenged mice to an intraperitoneal GTT (i.p. GTT) and an oral glucose gavage GTT (OGTT). Results showed that the glucose response was higher in *Sucnr1*- β KO mice than in control mice for both tests, which was accompanied by a reduced insulin secretory response (**Figure 5F**). These differences were not associated with changes in the glucose-associated GLP-1 response in *Sucnr1*- β KO mice on HFD (**Figure 5G**). Likewise, no appreciable changes were observed in insulin sensitivity directly measured by an insulin tolerance test (**Figure 5H**) or indirectly by assessing the homeostasis model assessment index of insulin resistance (**Figure 5I**). Altogether, these data demonstrate that SUCNR1 plays an essential role in insulin secretion in an *in vivo* obesity context.

Succinate functions as a hormone and is associated with the potentiation of insulin secretion in humans

To explore the physiological significance of succinate as an insulin secretagogue, we investigated the potential link between circulating succinate kinetics and pancreatic β -cell function in response to an OGTT and to an isoglycemic intravenous glucose infusion (IIGI) in a cohort of normal glucose tolerant (NGT) or non-NGT subjects according to the American Diabetes Association criteria (58) (**Figure 6A**, clinical and anthropometric data in **Table 1**). As expected, subjects in the non-NGT group had higher fasting plasma glucose and 2-hour glucose during the OGTT and were insulin resistant. Accordingly, these patients had higher glucose excursions during both OGTT and IIGI, which were accompanied by higher insulin and C-peptide responses (**Figure 6B**). Analysis of the insulin secretion rate (ISR) revealed no significant differences between groups during fasting, although an upward trend was observed in the non-NGT group (**Figure 6C**). Conversely, stimulated ISR was higher in the non-NGT group during both OGTT and IIGI (**Figure 6D**), which agrees with relative hyperglycemia and the characteristic hyperinsulinemia of insulin-resistant states. These differences were not associated with variations in the estimated β -cell glucose sensitivity (**Figure 6E**). Of note, the fasting levels of circulating succinate were significantly higher in the non-NGT group than in the NGT group (**Table 1**), which agrees with our previous findings that circulating succinate is an early biomarker of metabolic dysfunction (59, 60). No differences were found between groups in terms of the GLP-1 response (a recognized factor of the amplifying pathway) to the glucose challenge, independently of the route of glucose administration (**Figure 6F**), or the total incretin effect (**Table 1**). Strikingly, however, the succinate response to glucose,

which was found higher after nutrient passage through the gut (similar to GLP-1), was significantly higher in the non-NGT group than in the NGT group (**Figure 6G**).

According to the GLP-1 response (**Figure 6F**), incretin potentiation was similar between groups during the OGTT ($p=0.7118$) (**Figure 6H**). Contrastingly, during the IIGI, in which the incretin effect is considered negligible, the curve of glucose-induced potentiation exhibited a modest decrease in NGT individuals, while conversely, there was a slight increase in non-NGT individuals over time ($p<0.0001$) (**Figure 6I**). To determine whether succinate is associated with insulin secretion potentiation, we performed a correlation analysis between the potentiation ratio and the succinate response during the IIGI. A significant correlation between both variables was observed (**Figure 6J**). Overall, this analysis indicates that succinate potentiates insulin secretion, which may be particularly relevant for the hyperinsulinemia developed in insulin-resistant states.

DISCUSSION

Our study uncovers a hitherto unknown molecular link between the succinate/SUCNR1 axis and β -cell function. While previous research in this area primarily focused on the ability of cell-permeable succinate analogs to stimulate insulin secretion through TCA cycle-anaplerosis (13–16), our findings reveal that beyond to its intracellular functions, extracellular succinate stimulates insulin secretion in hyperglycemic states in mice and humans via SUCNR1 activation. This mechanism represents a novel amplifying pathway for insulin secretion, which may be relevant in prediabetic states where the oversecretion of insulin is an adaptive response to overcome insulin resistance.

Several GPCR-mediated mechanisms cooperate in β -cells to regulate insulin secretion (61). Our study establishes that SUCNR1 in β -cells potentiates insulin secretion in response to high glucose levels, similar to incretins such as GLP-1 and GIP (62). With regards to the transduction pathways underlying this insulinotropic effect, SUCNR1 signaling differs among cell types (52), but with a preference to engage G_i or G_q proteins (36, 63). We identified a G_q - and PKC-dependent mechanism linking SUCNR1 activation to the potentiation of insulin secretion. Some studies have speculated that increases in $[Ca^{2+}]_i$ observed upon SUCNR1 activation (which we also observed in β -cells) are mediated by G_i and $\beta\gamma$ -subunit-induced PLC- β activation rather than G_q (64, 65), but recent studies have established that G_q engagement is required for PKC and $[Ca^{2+}]_i$ -mediated responses upon SUCNR1 activation in immune

cells (36, 66). Likewise, other GPCRs such as GPR40 (activated by free fatty acids) have been reported to use pathways mediated by Gq, PKC, and $[Ca_{2+}]_i$, which are known to trigger insulin secretion (67, 68).

Our findings identify the succinate/SUCNR1 axis as a novel amplifying route for insulin secretion in response to hyperglycemia. Indeed, high glucose significantly increases β -cell sensitivity to extracellular succinate by elevating SUCNR1 expression. Accordingly, SUCNR1 expression is significantly greater in islets from donors with obesity and T2D than in lean control donors, which fits with our previous findings of higher circulating succinate levels in the former (59, 60). The upregulation of SUCNR1 associated with hyperglycemia differs, however, from the downregulation reported for GLP1-R in conditions associated with high glucose (69, 70). Notably, several identified risk variants for T2D have been mapped at the *SUCNR1* locus or are in close proximity to *cis*-regulatory elements (40, 42, 71). Collectively, these findings might suggest that modifications in SUCNR1 expression in β -cells in some diseases are linked to metabolic cellular adaptations and an elevated risk of developing T2D.

SUCNR1 activation is traditionally recognized as a mechanism bolstering tissue damage (72–77). Indeed, chronic elevation of extracellular succinate is found in low-grade inflammatory metabolic disorders such as cardiovascular disease, obesity, and T2D (26, 59, 78, 79). However, circulating succinate levels also rise transiently in response to normal physiological processes such as exercise (12) and food intake (33). The nutritional regulation of succinate is dependent on glucose sensing by the intestine and the metabolic status of the subject (33), suggesting a key signaling role for succinate for metabolic processes related to energy management (10). Along this line, we recently showed that the succinate/SUCNR1 axis is a novel regulator of leptin production in adipocytes (80). This study, in addition to our current findings identifying a novel function for succinate as an insulin secretagogue, strongly supports a role for SUCNR1 as part of the metabolite-sensing machinery allowing cells to coordinate energy homeostasis. Consistent with this hypothesis, we show that β -cell specific depletion of SUCNR1 in mice leads to an aberrant metabolic phenotype characterized by increased glucose intolerance and defects in insulin secretion under HFD feeding. As no significant variations in β -cell mass were detected, SUCNR1 may primarily influence the functionality of adult β -cells, rather than in their development or the compensatory changes in β -cell mass that occurs during obesity (81). However, *Sucnr1*- β KO mice on a HFD exhibited a trend towards reduced β -cell mass. While this observation is not definitive, it warrants the need for a more in-depth investigation into the role of SUCNR1 in β -cell proliferation and apoptosis,

to better understand its potential influence on β -cell mass adjustments in obesity. The observed similarities in the GLP-1 response between *Sucnr1*- β KO mice and control mice on a HFD, coupled with the pronounced impairment in insulin secretion and glucose tolerance in the *Sucnr1*- β KO mice, underscores the pivotal role of SUCNR1 in preserving insulin secretion. Despite the absence of incretin response both in *Sucnr1*- β KO mice and control mice in diet-induced obesity, β -cells in *Sucnr1*- β KO mice fall short in counteracting hyperglycemia, which emphasizes the critical involvement of the succinate-SUCNR1 axis in this scenario. Moreover, our findings align with an earlier study using global *Sucnr1* knockout mice, which exhibited progressive glucose intolerance under HFD and an impaired insulin response, occurring as part of a complex phenotype (25). However, other organs (e.g., adipose tissue) may also influence these phenotypes, underscoring the importance of analyzing the effects of SUCNR1 in specific cell types (10, 24, 80).

Our study in patients highlights the significance of the succinate/SUCNR1 axis in preserving glucose homeostasis during metabolic challenges. Prediabetes, a state observed in non-normal glucose tolerant (non-NGT) patients, is characterized by glucose intolerance resulting from systemic insulin resistance, which is substantially compensated for by the oversecretion of insulin (82) through mechanisms that remain elusive. When challenged with glucose, we found that individuals with abnormal glucose tolerance exhibited an amplified succinate response, while the GLP-1 response mirrored that of subjects with NGT. This suggests that factors beyond incretins may play a role in modulating the enhanced insulin secretion seen in prediabetic conditions. In this context, succinate emerges as a novel regulator of the observed hyperinsulinemic response in prediabetic patients. No significant differences in incretin-related potentiation were observed between the groups during an OGTT, likely due to preserved operation of the incretin system in patients with non-NGT. However, during intravenous glucose infusion, where the incretin effect on insulin secretion is negligible (83), we found a positive correlation between the succinate response and insulin secretion potentiation. The succinate response to glucose is higher during –but not exclusive to– the oral challenge (33), which prompts the consideration of other succinate sources in addition to the intestine. We observed that β -cells secrete more succinate when they are exposed to high glucose, which is consistent with previous studies showing that hyperglycemia can also lead to local increases in succinate (76, 84), likely due to overactivation of the TCA cycle (85). Thus, it is tempting to speculate that succinate endogenously produced in β -cells could be partly responsible for SUCNR1 engagement in an autocrine fashion. Succinate

nate derived from the gut microbiota is also a potential source (10), and indeed, other metabolites derived from the gut microbiota, including short-chain fatty acids, have been described as insulin secretagogue GPCR ligands (86, 87).

Our findings highlight SUCNR1 as a potential new therapeutic target to enhance insulin secretion in the context of T2D management. Indeed, the nutritional modulation of succinate is lost in patients with morbid obesity and T2D (33). Nonetheless, further research is needed due to the broad expression of SUCNR1 and its pleiotropic functions (10). While the succinate/SUCNR1 axis is crucial for the resolution of inflammation (24, 36), activation of SUCNR1 in an inflammatory context could be detrimental due to its pro-inflammatory effects in the acute phase of the immune response (74, 88). Along these lines, decreasing the higher levels of circulating succinate acting on its intestinal source, rather than directly activating SUCNR1, has recently emerged as a potential strategy for treating metabolic diseases (89). Whether this strategy could also be effective in recovering the function of SUCNR1 on insulin secretion deserves further analysis.

METHODS

Human cohort

The study was carried out at the Unit of Functional Studies, Institut d'Investigació Sanitària Pere Virgili, Hospital Universitari Joan XXIII, Tarragona, Spain. As part of a screening protocol for obesity in the general population, healthy asymptomatic subjects were selected from those who visited the Endocrinology Service. The main inclusion criteria were as follows: age between 18–65 years, BMI < 35 kg·m⁻²; absence of underlying pathologies (cancer, liver disease, kidney disease or systemic inflammation) on physical examination, diagnostic tests other than those associated with an excess of weight; and signing the protocol-informed consent. Additionally, inclusion criteria required a level of HbA1c < 6.5% and a fasting glucose level < 126 mg·dl⁻¹ in two separate occasions. After the initial triage, thirty subjects were selected to be submitted to an oral glucose tolerance test (OGTT) with 75 g of glucose. Individuals were classified according to the American Diabetes Association (ADA) criteria (58) as normal glucose tolerant (NGT; fasting glucose levels ≤ 110 mg·dl⁻¹ and a normal OGTT response) or non-NGT (fasting glucose levels ≥ 110 and < 126 mg·dl⁻¹, or impaired glucose tolerance or diabetes during an OGTT following the ADA criteria) (clinical information summarized in **Table 1**). The main exclusion criteria were pregnancy

and lactation; vegetarianism, veganism, or eating disorders; chronic treatment with anti-inflammatory agents, antibiotics, or cortisol; and major psychiatric antecedents, alcoholism, or drug abuse.

Assessment of β -cell function and succinate dynamics in humans

All participants underwent two procedures on separate occasions. First, we applied a 3-hour OGTT and subsequently a 3-hour intravenous isoglycemic glucose infusion (IIGI), reproducing the plasma glucose time curve recorded during the OGTT using an *ad hoc* algorithm. Plasma glucose was determined every ten minutes during both procedures, whereas insulin, C-peptide, GLP-1, and succinate were determined in plasma samples at -30, 0, 10, 20, 30, 60, 90, 120, 150, and 180 minutes. Body composition was determined by electric bioimpedance (Tanita Europe BV; Amsterdam, The Netherlands). Plasma lipid, hepatic and renal profiles were determined by standard enzymatic methods. Plasma glucose was determined by the glucose oxidase method (GM-9; Analox, London, UK). Plasma insulin and C-peptide levels were determined by an immunochemiluminometric assay (ADVIA Centaur; Siemens Healthcare, Erlangen, Germany). Total plasma GLP-1 levels were determined by ELISA (EZGLP1T-36 K; Merck, Darmstadt, Germany). Plasma succinate was determined in plasma filtrates (10 kDa) using a fluorometric assay (EnzyChrom Succinate Assay Kit; BioAssay Systems, Hayward, CA).

The areas under the curve (AUC) were calculated using the trapezoid rule. Insulin sensitivity was estimated from the plasma glucose and insulin responses to oral glucose using the OGTT-derived index of insulin sensitivity (OGIS) (90). *In vivo* insulin secretion, β -cell function parameters, and the incretin effect were calculated by simultaneous modeling analysis of the IIGI and the OGTT, as previously described (91). The model reconstructs insulin secretion from plasma C-peptide using an established model of C-peptide kinetics (92) and it extends a widely used model for the analysis of the OGTT (93). In brief, the model describes the relationship between insulin secretion and glucose concentration during the IIGI as a quasi-linear function (i.e., the dose-response). This response is modulated during the test by a time-dependent factor, which averages to one. This time-dependent factor, denoted as glucose-induced potentiation, accounts for potentiation phenomena that typically prompt glucose-dependent insulin secretion higher at the end of the test compared to the beginning. The model also includes a term describing early secretion, related to first-phase insulin release. Insulin secretion during the OGTT is

described by the same terms, although the dose-response is multiplied by another time-dependent factor, typically greater than one, which accounts for the effect of incretin hormones on insulin secretion. This time-dependent factor is denoted incretin potentiation, and its AUC is a measure of the total incretin effect. To study a potential relationship between the insulin secretion potentiation during the IIGI and succinate dynamics, we used the potentiation ratio between 2 hours and the baseline, and the corresponding 2-hour incremental succinate AUC mean.

Human islets

Human islets for gene expression analysis were isolated from the pancreas of non-diabetic adult brain-dead organ donors at Hospital Universitari de Bellvitge and Institut d'Investigació Biomèdica de Bellvitge (Barcelona, Spain) as previously described (94).

Human islets for protein expression assays were obtained from the Integrated Islet Distribution Program and Prodo Laboratories (Aliso Viejo, CA, USA) and processed as previously detailed (95, 96).

Animals

Male Wistar rats (Charles River Laboratories, Wilmington, MA) were housed in the Animal Facility of Miguel Hernández University. All mice were housed in the Animal Facility of the Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili. Housing conditions consisted of automated 12-h light-dark cycles at 20–22°C, 40–60% relative humidity, and *ad libitum* access to NCD (3.1 kcal% fat; SAFE Diets, A04) or HFD (60 kcal% fat; Research Diets Inc., D12492). HFD availability to mice started at 8 weeks of age until the study endpoint. Male mice were weighed prior to any experimental procedure. At the end of the study, mice were fasted for 16 h and then euthanized by cervical dislocation. Tissues were collected and immediately snap-frozen in liquid N₂ or fixed in 4% formaldehyde.

Male wildtype C57BL/6 mice and male diabetic *db/db* mice were obtained from Charles River Laboratories. β -cell specific *Sucnr1* knockout mice (*Sucnr1*^{fl/fl} *Ins1*^{Cre/+}, *Sucnr1*- β KO) were obtained in the F2 generation by crossing parental homozygous *Sucnr1* floxed mice (*Sucnr1*^{fl/fl} *Ins1*^{+/+}, control) (24) and *Ins1*^{Cre/+} mice, the latter kindly provided by J. Ferrer (Centre for Genomic Regulation, Barcelona, Spain). Briefly,

Ins1^{Cre/+} mice were generated by a knock-in strategy into one endogenous *Ins1* allele and present more specificity for restricted β -cell Cre expression, avoiding recombination in other tissues, including the brain (57). Control Cre-negative mice were generated in the same litters along with *Sucnr1*- β KO mice (**Supplemental Figure 1**). All resulting mice were generated on a pure C57BL/6 background and genotyped by PCR using specific primers to amplify *Sucnr1*⁺ and *Sucnr1*^f alleles (300 bp product for the wild-type allele and 450 bp product for the floxed form) and to amplify *Ins1*⁺ or *Ins1^{Cre}* alleles (524 bp for the wild-type and 865 bp for the mutated structure).

Islet isolation, FACS purification and cell culture of rat α - and β -cells

Rat islets were isolated by collagenase digestion and handpicked. Islets were dissociated into single cells by mechanical and enzymatic dispersion using trypsin (1 mg·ml⁻¹; Sigma-Aldrich, Saint Louis, MO) and DNase I (1 mg·ml⁻¹; Roche, Basel, Switzerland) for 5 min at 31°C under agitation. Dissociated cells were resuspended in Earle's medium (0.79 mM NaH₂PO₄, 0.8 mM MgSO₄, 2.4 mM CaCl₂, 115 mM NaCl, 5.4 mM KCl) containing 2.8 mM glucose. FACS sorting of β and α -cells was done using a FACS Aria III cell sorter (BD Biosciences, Franklin Lakes, NJ), as described (97). The percentage of viability was determined after incubation with the DNA-binding dyes propidium iodide (5 μ g·ml⁻¹; Sigma-Aldrich) and Hoechst 33342 (5 μ g·ml⁻¹; Sigma-Aldrich). A minimum of 600 cells were counted in each experimental condition. Viability was evaluated by two independent observers, one of them blinded to sample identity. The agreement between observers was > 90% (97). After sorting, purified β -cells were cultured in Ham's F-10 medium containing 10 mM glucose, 2 mM Glutamax (Thermo Fisher Scientific, Waltham, MA), 0.5% bovine serum albumin fraction V (BSA; Sigma-Aldrich, Saint Louis, MO) 50 μ M IBMX (Sigma-Aldrich), 50 units·ml⁻¹ penicillin, 50 μ g·ml⁻¹ streptomycin (Thermo Fisher Scientific) and 5% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific); α -cells were cultured in the same medium but including 6.1 mM glucose and 10% FBS. The purity of the α and β -cell preparations was evaluated by immunofluorescence and was calculated as a percentage of positive cells for each cell type (98). α - and β -cells were immunostained with mouse monoclonal anti-insulin or anti-glucagon antibodies (both from Sigma-Aldrich) for 1 h followed by incubation with a rabbit anti-mouse secondary antibody conjugated with Alexa Fluor 488 (A32723) or Alexa Fluor 568 (A11031) from (Thermo Fisher Scientific).

Physiological experiments on mice

For the i.p. and oral GTT, 16- and 54-week-old male mice were fasted for 16 h, and blood glucose was measured before and 15, 30, 60, and 120 min after administration of glucose ($2 \text{ g} \cdot \text{kg}^{-1}$ body weight). For the ITT, mice were fasted for 4 h, and blood glucose was measured before and 15, 30, 60, and 120 min after an i.p. injection of recombinant human insulin ($0.75 \text{ IU} \cdot \text{kg}^{-1}$ body weight; Actrapid, Novo Nordisk, Denmark). Blood glucose was measured using a glucose meter (Accu-Chek; Roche). The area under the curve for glucose was calculated using the trapezoidal rule for any tolerance test (99). Plasma insulin was determined using a Mouse Insulin Ultrasensitive ELISA kit (Mercodia, Uppsala, Sweden). GLP-1 levels were analyzed in plasma using a Mouse GLP-1 ELISA kit (Crystal Chem, Elk Grove Village, IL).

Pancreas histology and immunohistochemistry

Pancreatic tissue was obtained from an autopsied adult male and mice. Tissues were washed with PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C . Tissue samples were then dehydrated and degreased before paraffin embedding, and tissue sections ($2 \mu\text{m}$ thickness) were stained with H&E.

For immunohistochemistry, formalin-fixed paraffin blocks of pancreatic tissue were sectioned at a thickness of $4 \mu\text{m}$, and each slide was deparaffinized in xylene for 20 min, rehydrated with a decreasing ethanol series and washed with PBS. Sections were heated at 96°C for 20 min and then incubated for 30 min with a primary antibody against SUCNR1 (1:2 dilution, NLS3476; Novus Biologicals, Centennial, CO) (100) and chromogranin A (Clone DAK-A3; Agilent, Santa Clara, CA). Automatic immunodetection was performed using the EnVision FLEX method (Agilent) with 3,3'-diaminobenzidine chromogen as substrate and hematoxylin counterstain. Panoramic 250 Flash III DX Scanner and SlideViewer 2.6 software (3DHISTEC, Budapest, Hungary) were used for image scanning, visualization, and image acquisition of stained pancreatic tissues (101).

Pancreas morphometric analysis

Samples were collected and fixed in 4% formaldehyde at 4°C for 16 hours, followed by paraffin embedding. For morphometric analysis, at least 3 non-consecutive ($150\text{-}\mu\text{m}$ apart) $3\text{-}\mu\text{m}$ -thick sections were obtained for each pancreas region (head,

body, and tail) and analyzed per mouse. Sections were subjected to immunostaining. Briefly, slides were heated, in 10 mM sodium citrate, followed by blocking with donkey serum and incubation with primary antibodies detecting insulin (sc-8033, Santa Cruz Biotechnology, Dallas, TX) and glucagon (SAB4501137; Sigma-Aldrich). Specific signals were detected with fluorochrome-conjugated secondary antibodies with Alexa Fluor 488 (A11029) or Alexa Fluor 568 (A21043) from Thermo Fisher Scientific. Slides were counterstained with DAPI (Sigma-Aldrich) to visualize nuclei. Images were captured using a Zeiss Axio Vert A1 fluorescence microscope system (Zeiss, Oberkochen, Germany). β -cell mass was calculated by generating the ratio of the cross-sectional area of the total number of pixels from insulin-positive cells to the cross-sectional area of the total number of pixels of pancreatic tissue revealed by H&E staining, multiplied by the pancreas weight of the mouse. β -cell mass was calculated using a similar approach but using the total number of pixels from glucagon-positive cells. Images were processed and analyzed using Fiji 2.9.0 software.

Cell lines

EndoC- β H1 is an engineered cell line obtained from a female human pancreas subjected to lentiviral transfection of the SV40 large T antigen (SV40LT) expressed under the control of the insulin promoter and the human telomerase reverse transcriptase (hTERT) (102). These cells present several similarities to primary human β -cells (103). EndoC- β H1 cells were cultured as described (95). Briefly, before seeding the cells, the culture dishes and plates were coated with a mixture of DMEM (4.5 g·l⁻¹ glucose; Thermo Fisher Scientific, Waltham, MA), fibronectin (2 μ g·ml⁻¹; Thermo Fisher Scientific), and extracellular matrix (1%; Sigma-Aldrich, Saint Louis, MO). The coated flasks were then incubated for at least 1 hour in a 5% CO₂ atmosphere at 37°C. EndoC- β H1 cells were maintained at 37°C and 5% CO₂ in DMEM-based media consisting of 1 g·l⁻¹ glucose, 2% BSA, 50 μ M 2-mercaptoethanol (Sigma-Aldrich), 10 mM nicotinamide (Sigma-Aldrich), 5.5 μ g·ml⁻¹ transferrin (Sigma-Aldrich), 6.7 ng·ml⁻¹ sodium selenite (Sigma-Aldrich), and 1% penicillin-streptomycin (Thermo Fisher Scientific). To measure SUCNR1 protein levels, cells were starved for 16 h in 2.8 mM glucose DMEM-based media followed by treatment with 5.5, 11.1, 16.7, or 25 mM glucose for 24 h.

The latest iteration of the human EndoC cell series is the EndoC- β H5 cell line, which was purchased from Human Cell Design (Toulouse, France). According to the supplier, these cells are derived from a female fetus and demonstrate an improved in-

sulin secretion response, heightened sensitivity to incretins, and more pronounced expression of β -cell identity markers in comparison with other human β -cell lines. The generation process of these cells was similar to previous models, but the SV40LT and hTERT transgenes were removed through Cre-mediated recombination to produce mature, non-proliferative cells. EndoC- β H5 cells were seeded at 1×10^5 cells per well in β COAT matrix (Human Cell Design) pre-treated 96-well Cell⁺ plates (Sarstedt, Nümbrecht, Germany) and cultured for 10 days prior to performing the GSIS assays. Cells were maintained in ULTI- β 1 medium (Human Cell Design), and the medium was changed 4 hours after seeding, when all viable cells had attached, and then again 4 days later.

MIN6 is an insulinoma-derived cell line from transgenic male mice transfected with SV40 T-antigen, with the ability to secrete insulin in the presence of glucose (104). MIN6 cells were maintained under standard culture conditions (Miyazaki et al., 1990). Briefly, MIN6 cells were cultured at 37°C and 5% CO₂, in DMEM consisting of 4.5 g·l⁻¹ D-glucose, 2 mM L-glutamine (Thermo Fisher Scientific), 15% fetal bovine serum (Thermo Fisher Scientific), 1% penicillin-streptomycin, 20 mM HEPES (Thermo Fisher Scientific), and 50 μ M 2-mercaptoethanol. To assess *Sucnr1* gene expression levels, cells were starved for 16 h in 2.8 mM glucose DMEM-based media followed by stimulation with 5.5, 11.1, or 16.7 mM glucose for 3 and 24 hours.

Gene expression analysis

RNA was extracted from tissues or cells using TRIzol Reagent and cDNA was synthesized using the High-capacity cDNA Reverse Transcription kit (both from Thermo Fisher Scientific). Quantitative real-time PCR amplification was performed on a 7900HT Fast Real-Time PCR System with the TaqMan Gene Expression Assay from Thermo Fisher Scientific (probes are listed in the Key Resources Table). Relative mRNA levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method, corrected for the corresponding housekeeping gene expression levels.

Western blotting

Cells or tissues were lysed in M-PER buffer containing Halt Protease and Phosphatase Inhibitor Cocktails and protein concentration was measured using the BCA protein assay kit (all from Thermo Fisher Scientific). Samples were resolved on SDS-PAGE gels and proteins were transferred onto PVDF membranes (Merck KGaA, Darm-

stadt, Germany). Membranes were blocked in a blocking buffer consisting of 5% blotting-grade blocker non-fat dry milk (Bio-Rad, Hercules, CA) and incubated with primary antibodies overnight at 4°C. A polyclonal primary antibody against SUCNR1 (NBP1-0086) was obtained from Novus Biologicals (Centennial, CO). A monoclonal anti- β -actin antibody was purchased from Sigma-Aldrich (A1978). Primary antibodies against phospho-PKC δ (T505) (#9374), phospho-PKC ζ/λ (T410/403) (#9378), phospho-Akt (S473) (#4058), phospho-GSK-3 α/β (S21/9) (#9331), phospho-ERK1/2 (T202/Y204) (#9101), phospho-p38 (T180/Y182) (#4511), and phospho-CREB (S133) (#9198) were obtained from Cell Signaling Technology (Danvers, MA). Membranes were incubated with the respective HRP-linked secondary antibodies for 1 h and developed using the Pierce ECL Western Blotting Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Band intensity was captured using the iBright CL1000 Imaging System and analysis was performed with the iBright Analysis Software (Thermo Fisher Scientific).

Succinate secretion assay and quantification

MIN6 cells were washed twice in Krebs-Ringer-Phosphate-HEPES (KRPH) buffer (5 mM Na₂HPO₄, 1 mM MgSO₄, 1 mM CaCl₂, 136 mM NaCl, 4.7 mM KCl, 20 mM HEPES pH 7.4 and 0.5% BSA), and then incubated for 1 h in KRPH buffer supplemented with 2.8 mM glucose. The buffer was then replaced with KRPH buffer containing 2.8 or 16.7 mM glucose, for 2 h. The CM was collected at the end of the assay was centrifuged at 200 rpm for 15 min at 4°C and the supernatant was used to quantify succinate content using the fluorometric EnzyChrom Succinate Assay Kit (BioAssay Systems).

Glucose-stimulated insulin secretion assay

MIN6 cells were washed twice in KRPH buffer, incubated in KRPH buffer with 2.8 mM D-glucose for 1 h at 37°C, 5% CO₂ and saturated humidity, and then washed twice with KRPH buffer. Cells were then incubated for 1 h in KRPH buffer containing either 2.8 or 16.7 mM D-glucose and the corresponding stimulus. EndoC- β H5 cells were subjected to GSIS assays following the manufacturer's protocol (Human Cell Design). In brief, cells were starved in ULTI-ST medium for 24 hours, then washed twice in β Krebs medium (both from Human Cell Design) supplemented with 0.1% BSA (β Krebs-BSA) and incubated in β Krebs-BSA medium for 1 h at 37°C, 5% CO₂ and saturated humidity. Cells were then stimulated for 40 min with 0 or 20 mM glucose and the indicated stimulus in β Krebs-BSA media.

Reagents for GSIS assays were 0.5–1 mM disodium succinate (W327700; Sigma-Aldrich), 50 μ M *cis*-epoxysuccinic acid (cESA; E0449; TCI Chemicals, Tokyo, Japan), 1 μ M NF-56-EJ40 (HY-130246; MedChemExpress, Monmouth Junction, NJ), 1 μ M Gö 6983 (#2285, Tocris, Bristol, UK), and 1 μ M FR900359 (#33666, Cayman Chemical Co., Ann Arbor, MI). Inhibitors were pre-incubated for 3 h in all media preparations during the assays at the reported concentrations. At the end of GSIS assays, the CM was collected and centrifuged at 300 RCF for 15 min at 4 °C, and the supernatant was stored at -80°C. The remaining cells were washed once in PBS and lysed with M-PER buffer with Halt Protease Cocktail (Thermo Fisher Scientific) for protein quantification. The insulin content in the CM was measured using Mouse or Human Insulin ELISA kits (Mercodia).

Measurement of cytosolic Ca^{2+}

Intracellular free Ca^{2+} was determined in MIN6 cells grown on glass coverslips (Menzel-Gläser, Germany). At 80% confluency, the coverslip was carefully transferred to a Petri dish containing 3 ml of Locke-HEPES buffer (LH) comprising 120 mM NaCl, 10 mM KCl, 15 mM NaHCO_3 , 3.3 mM MgCl_2 , 2.6 mM CaCl_2 , 2.8 mM D-glucose and 10 mM HEPES, pH 7.4 and including 6 μ M Fura-2 acetoxymethyl ester (Thermo Fisher Scientific). Cells were incubated at 37°C for 30 min in a cell incubator. For fluorescence recordings, the coverslip was carefully rinsed in LH, mounted in a specific holder (coverslip accessory L2250008; PerkinElmer, Waltham, MA), and placed in a quartz cuvette containing 1.3 ml of LH buffer. Measurements were made at 37°C with continuous mild stirring in an LS50B PerkinElmer fluorescence spectrometer equipped with a fast-filter accessory for Fura-2 fluorescence ratio measurements. Emission data (510 nm) were collected with alternate excitation at 340 and 380 nm and the ratio F_{340}/F_{380} was calculated in real-time using proprietary software (FL WinLab 2.0; PerkinElmer). The mobilization of cytosolic Ca^{2+} by extracellular succinate was evaluated before and after the increase of glucose to 16.7 mM.

Statistical analysis

The data presented in tables and graphics are displayed as either the mean $\pm$ SD or mean $\pm$ SEM, as indicated. In the box-and-whisker plots, the median is indicated by a center line, while the upper and lower quartiles are represented by the box limits, and the minimum to maximum range of the data set is indicated by the whis-

kers. The Shapiro-Wilk normality test was applied before any contrast hypothesis testing. When the data were normally distributed, a two-tailed Student's *t*-test was used to compare differences between two groups, applying Welch's correction if a difference between the variations of the compared groups was observed, whereas a one-way ANOVA with Dunnet's *post hoc* test was used to compare multiple groups to a control group. For time-course experiments, a two-way ANOVA with Bonferroni's correction for multiple comparisons were conducted. In cases where the data did not follow a normal distribution, the non-parametric Mann-Whitney two-tailed *U*-test for unmatched pairs or the Wilcoxon signed-rank test for matched pairs were used to compare differences as appropriate. Pearson's or Spearman's correlations were employed to calculate linear associations between continuous variables. Both GraphPad Prism 9 and RStudio software for Mac OS and IBM SPSS 27 software for Windows OS were utilized for the statistical analyses, and a *p*-value of less than 0.05 was considered statistically significant.

Study approval

The conduct of the study protocols was in accordance with the ethical principles outlined in the Declaration of Helsinki and received authorization from the relevant local ethics committees (IISPV Ethics Research Committee reference number 204/2017). Prior to their participation, all subjects were given a thorough explanation of the study protocol and provided written informed consent.

The use of human islets for gene expression analysis was approved by the local Ethics Committee of Hospital Universitari de Bellvitge (approval PR239/13), and signed consent was obtained from donor relatives. All studies and protocols involved in human islet procurement and experimentation (for the protein expression analysis) were approved by the Joslin Diabetes Center's Committee on Human Studies (approval CHS#5-05).

All animal studies conformed to the ARRIVE guidelines and were carried out under the supervision and approval of the Animal Welfare and Governmental Ethics Committee of the Universitat Rovira i Virgili (project reference number 10970). All experimental procedures involving animals conformed to the European Union Directive 2010/63/EU and the European Commission Recommendation 2007/526/EC on the protection of animals used for experimental and other scientific purposes, enacted under the Spanish Royal Decrees 53/2013 and 118/2021.

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AUTHOR CONTRIBUTIONS

Conceptualization was the responsibility of J.S.B., B.A., J.V., and S.F.V.; Methodology was the responsibility of J.S.B., B.A., M.E., J.B., M.R.D.D., D.F.D.J., L.M., R.B., and F.S.; Formal Analysis was conducted by J.S.B., B.A., J.B., F.S., A.T., and A.M.; Investigation was conducted by J.S.B., B.A., M.E., M.R.D., J.B., C.N.R., L.M., D.F.D.J., L.M., and R.B.; Resources were provided by L.M., A.T., A.M., and R.N.K.; Original draft writing was conducted by J.S.B.; Manuscript review and editing was the responsibility of J.S.B., A.T., A.M., R.N.K., J.V., and S.F.V.; Supervision was carried out by J.V. and S.F.V.; Funding was acquired by J.V. and S.F.V.

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Table 1. Clinical and Anthropometric Data of the Subjects Included in the Study

	Normal glucose tolerant (NGT)	Non-normal glucose tolerant (non-NGT)	<i>p</i>
n (female/male)	12 (9/3)	18 (8/10)	
Age (years)	36.2 ± 12.7	45.6 ± 11.0	0.039
BMI (kg·m ⁻²)	26.3 ± 2.8	30.5 ± 3.5	<0.001
Waist-to-hip (cm)	0.86 ± 0.07	0.94 ± 0.10	0.028
Fat mass (%)	31.5 ± 7.3	33.9 ± 6.8	0.373
FPG (mg·dl ⁻¹)	94.9 ± 4.8	106.0 ± 10.2	<0.001
Uric acid (mg·dl ⁻¹)	4.3 ± 1.0	5.8 ± 1.2	0.010
Total cholesterol (mg·dl ⁻¹)	155.8 ± 32.5	182.6 ± 36.4	0.049
HDL (mg·dl ⁻¹)	53.3 ± 10.1	51.7 ± 11.5	0.686
LDL (mg·dl ⁻¹)	86.5 ± 23.2	111.8 ± 32.0	0.026
Triglycerides (mg·dl ⁻¹)	80.4 ± 33.4	114.6 ± 64.6	0.084
AST (U·l ⁻¹)	17.8 ± 3.7	34.2 ± 22.4	0.002
ALT (U·l ⁻¹)	15.0 ± 4.3	27.2 ± 10.5	<0.001
GGT (U·l ⁻¹)	15.8 ± 7.0	58.9 ± 64.8	0.002
HbA1c (%)	5.1 ± 0.3	5.4 ± 0.3	0.005
2-h glucose (mg·dl ⁻¹)	120.4 ± 15.4	154.1 ± 40.5	0.003
Fasting insulin (pmol·l ⁻¹)	49.1 ± 14.6	64.9 ± 31.0	0.072
Fasting C-peptide (nmol·l ⁻¹)	0.33 ± 0.08	0.47 ± 0.19	0.030
Fasting GLP-1 (pmol·l ⁻¹)	24.0 ± 7.7	31.6 ± 17.7	0.121
Fasting Succinate (μmol·l ⁻¹)	47.8 ± 22.6	60.8 ± 23.3	0.142
OGIS (ml·min ⁻¹ ·m ⁻²)	406.6 ± 30.7	331.3 ± 51.0	<0.001
Incretin effect (a.u.)	1.78 ± 0.66	1.43 ± 0.36	0.114

Data are presented as mean ± SD. *p*-values were determined by the Student's *t*-test (with Welch's correction if SD is inconsistent between groups) or the Mann-Whitney U-test according to the data distribution. BMI: body mass index; FPG: fasting plasma glucose; HDL: high-density lipoprotein; LDL: low-density lipoprotein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; HbA1c: glycated hemoglobin; GLP-1: glucagon-like peptide 1; OGIS: OGTT-derived insulin sensitivity index; a.u.: arbitrary units.

FIGURES

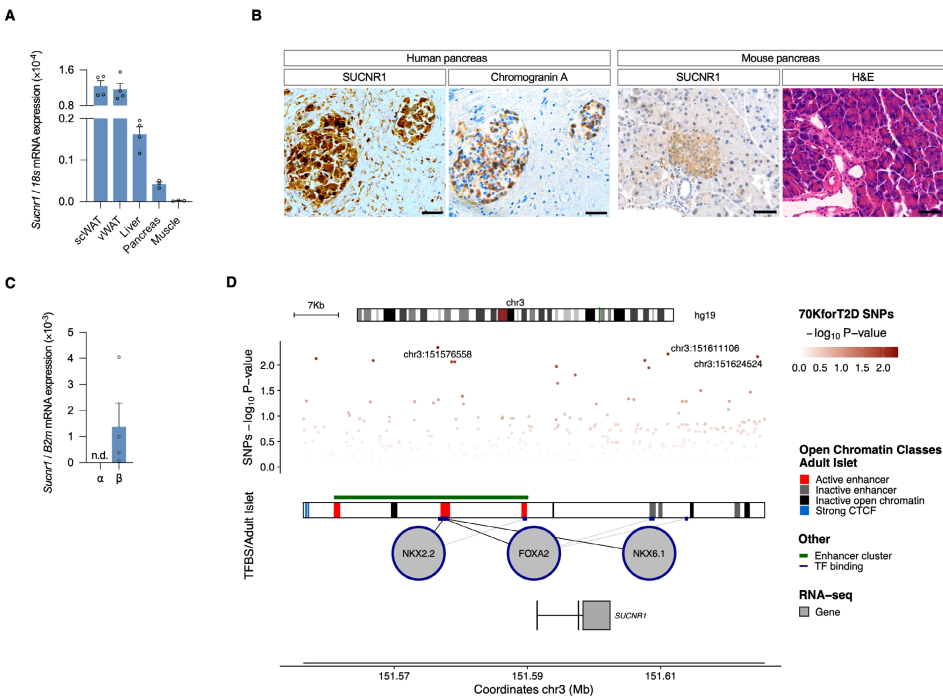


Figure 1: SUCNR1 is expressed in islets and β -cells. (A) *Sucnr1* mRNA levels analyzed in subcutaneous white adipose tissue (scWAT), visceral WAT (vWAT), liver, pancreas, and muscle tissue by qPCR (n=3-4). (B) Immunohistochemical (IHC) staining of SUCNR1 in human and mouse pancreas sections, and chromogranin A IHC staining or hematoxylin and eosin (H&E) staining. Scale bars represent 50 μ m. (C) Analysis of *Sucnr1* mRNA expression in α - and β -cells isolated by fluorescence-activated cell sorting (FACS) from rat islets (n=4). (D) *In silico* study of *SUCNR1* gene expression regulation by genomic sequences and specific human adult islet transcriptional factors, and single nucleotide polymorphisms (SNPs) associated with type 2 diabetes (T2D) localized within or surrounding the *SUCNR1* locus. Data are presented as mean $\pm$ SEM.

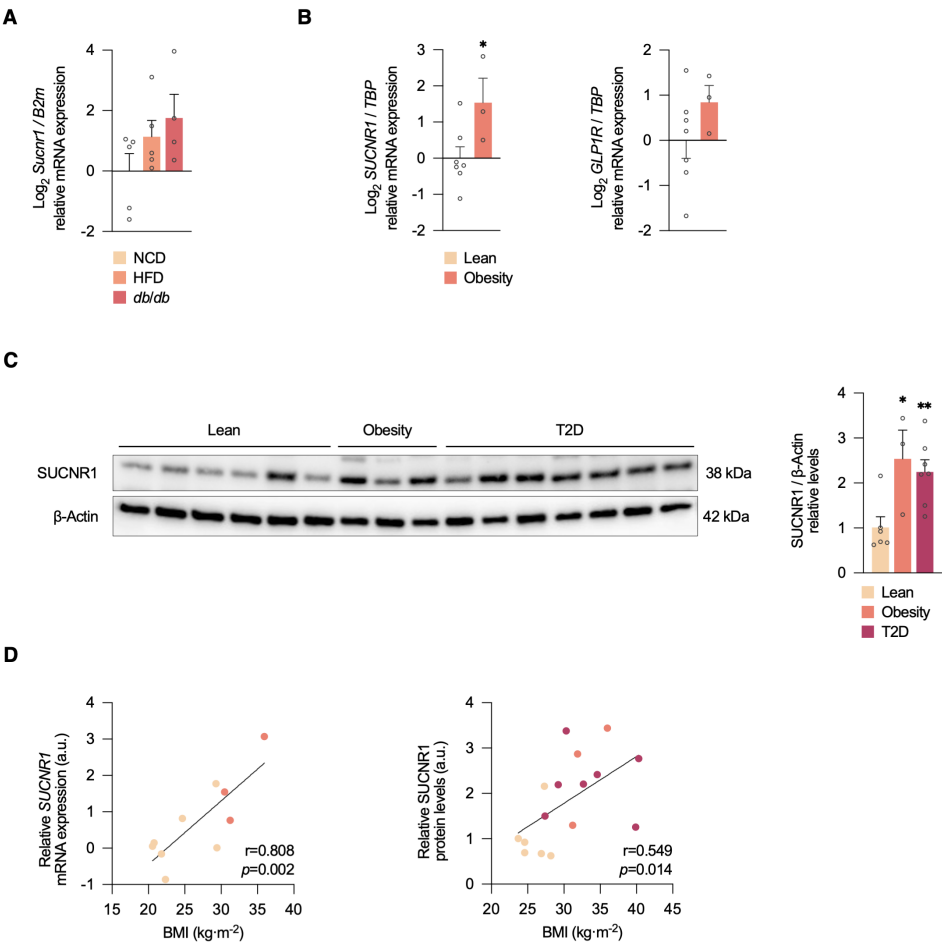


Figure 2: SUCNR1 levels in islets are dysregulated in obesity and type 2 diabetes. (A) *Sucnr1* mRNA expression in the entire pancreas of normal-chow diet (NCD)- and high-fat diet (HFD)-fed wild-type mice, and *db/db* mice on NCD (n=4-5). (B) *SUCNR1* and *GLP1R* mRNA expression in islets from healthy (n=7) and donors with obesity (n=3). (C) *SUCNR1* protein levels in human islet lysates from healthy (n=6) and donors with obesity (n=3) and T2D (n=7). (D) Linear correlations between the body mass index (BMI) of donors and *SUCNR1* mRNA (n=10) and protein (n=16) expression. Data are presented as mean $\pm$ SEM. * $p<0.05$ vs control (Student's *t*-test in (A) and (B) or Pearson's correlation coefficient in (D)).

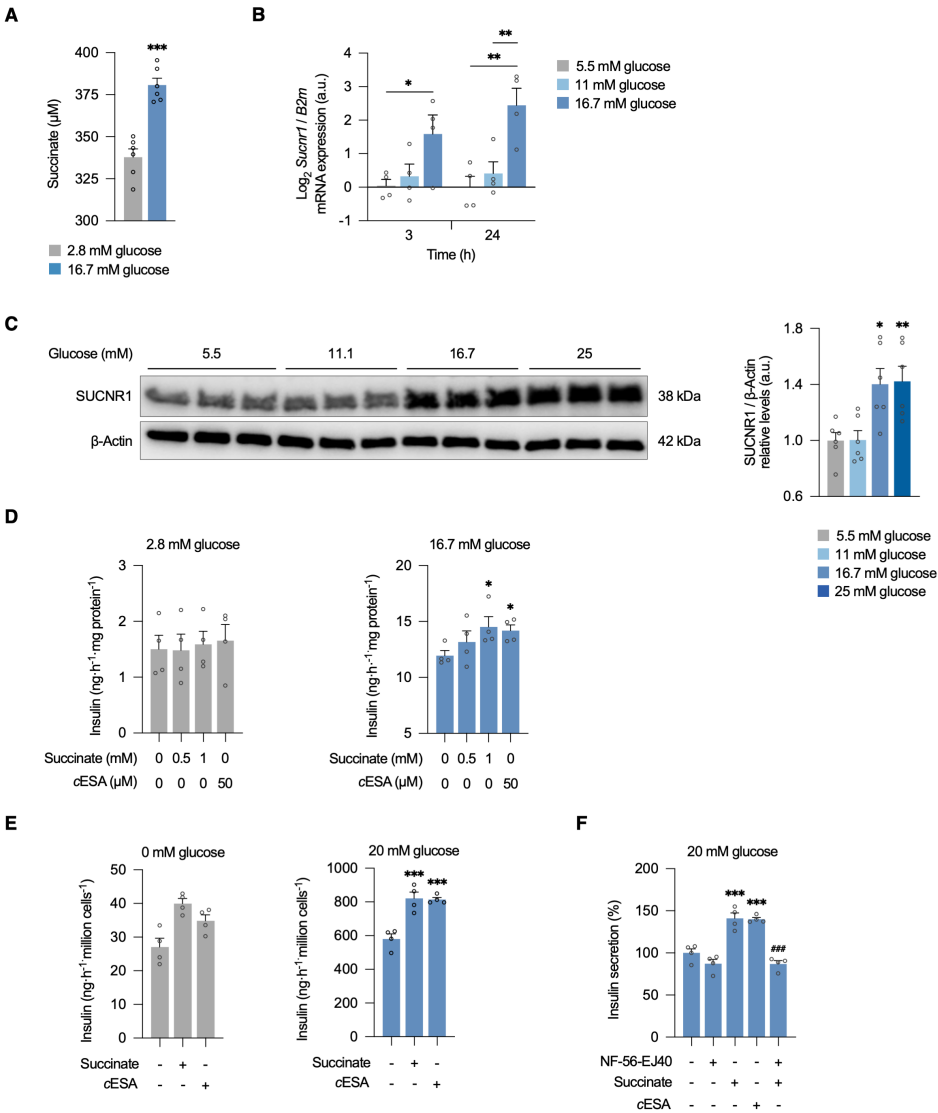


Figure 3: The Succinate/SUCNR1 axis enhances glucose-stimulated insulin secretion in β -cells. (A) Succinate quantification in the conditioned media (CM) of MIN6 cells cultured in low- or high-glucose conditions (n=4). (B) *Sucnr1* mRNA expression in MIN6 cells stimulated with different concentrations of glucose for 3 or 24 hours (n=4). (C) SUCNR1 protein levels in EndoC- β H1 cells stimulated with different concentrations of glucose for 24 hours (n=6). (D) Insulin quantification in the CM of MIN6 cells stimulated with succinate or *cis*-epoxysuccinic acid (cESA) at 2.8 mM or 16.7 mM glucose (n=4). (E) Insulin secretion in glucose-stimulated insulin secretion (GSIS) assays in Endo-C β H5 cells stimulated with 500 μM succinate or 50 μM cESA at 0 mM or 20 mM glucose, determined in the CM by ELISA (n=4). (F) Insulin

secretion in Endo-C β H5 cells incubated with a human-specific SUCNR1 antagonist (1 μ M NF-56-EJ40) and 500 μ M succinate or 50 μ M cESA (n=4). Data are presented as mean $\pm$ SEM. * p <0.05, ** p <0.01, *** p <0.001 vs basal conditions; ### p <0.001 vs succinate (Student's t -test in **(A)** or ANOVA with Dunnett's test for multiple comparisons in **(B-E)**).

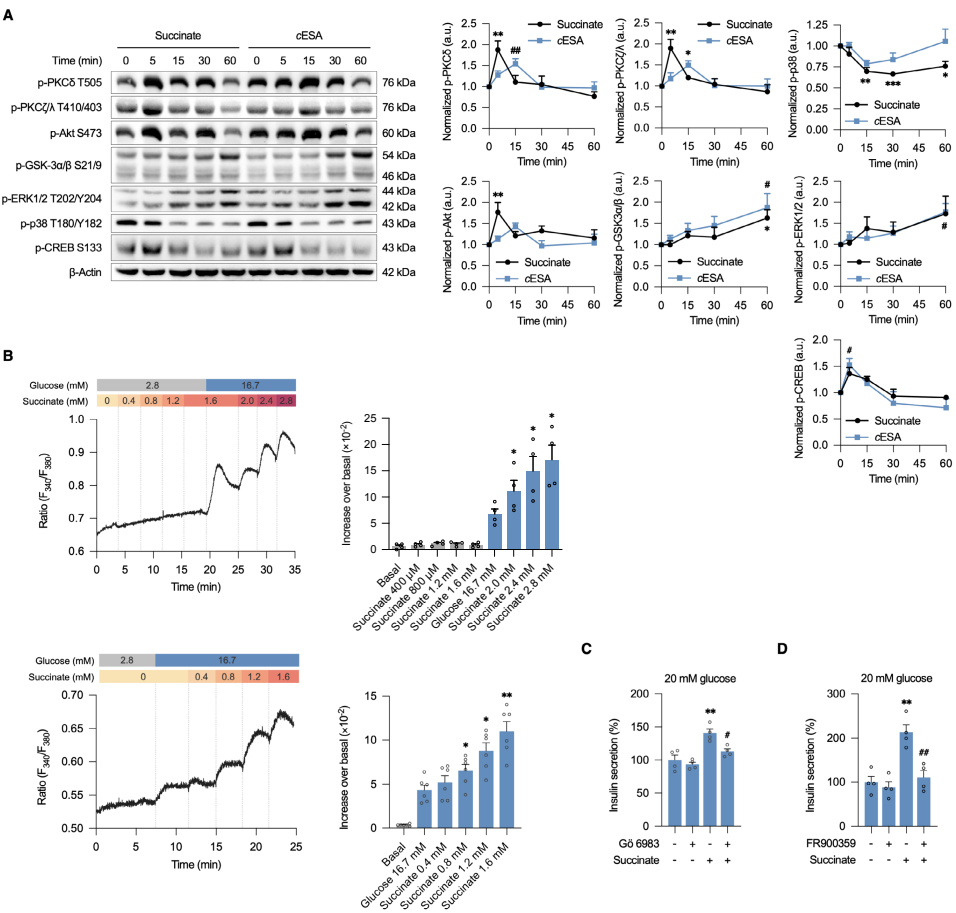


Figure 4: SUCNR1 activation in β -cells induces proximal signaling and Ca^{2+} mobilization, and is dependent on Gq and PKC pathways in β -cells. (A) Western blot analysis of several phosphoproteins in MIN6 cells stimulated with 500 μM succinate or 50 μM *cis*-epoxysuccinic acid (cESA) at different time points (n=4-5). (B) Calcium mobilization in MIN6 cells stimulated with glucose and succinate assessed by the Fura-2 fluorometric ratio (n=4-6). (C) Insulin secretion in EndoC- β H5 cells incubated with 500 μM succinate and 1 μM of the PKC inhibitor Gö 6983 (n=4). (D) Insulin secretion in EndoC- β H5 cells incubated with 500 μM succinate and the 1 μM of Gq inhibitor FR900359 (n=4). Data are presented as mean $\pm$ SEM. * p <0.05, ** p <0.01, *** p <0.001 when comparing succinate with the basal condition; # p <0.05, ## p <0.01, when comparing cESA with the basal condition, or an inhibitor with succinate (ANOVA with Dunnett's test for multiple comparisons in (A) and (B) or ANOVA with Tukey's test for multiple comparisons in (C) and (D)).

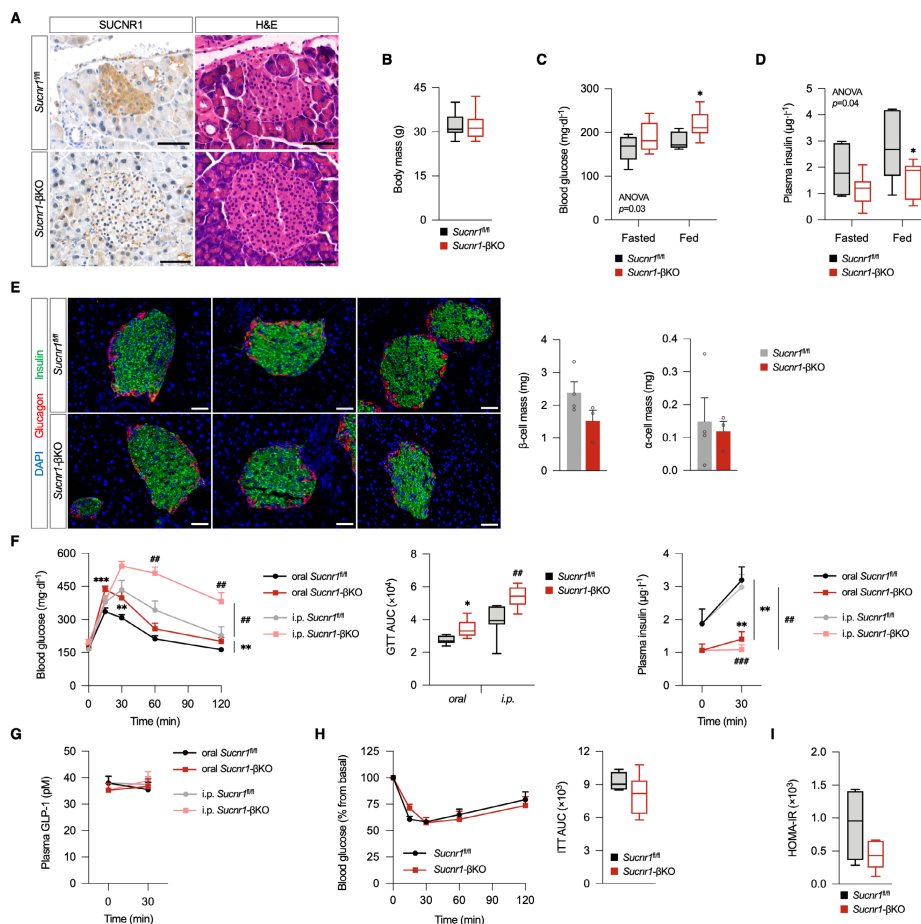


Figure 5: SUCNR1 in β -cells is required for preserving insulin secretion and glucose homeostasis in high-fat diet-fed mice. (A) Immunohistochemical staining of SUCNR1 in pancreas sections of control and *Sucnr1-βKO* mice accompanied with serial H&E staining. Scale bar represents 50 μ m. (B) Body mass of control and *Sucnr1-βKO* mice under HFD for 8 weeks (n=8-9). (C) Morphometric analysis of control and *Sucnr1-βKO* mice by immunofluorescence staining with insulin and glucagon and counterstained with DAPI (n=3-4). Scale bar indicates 50 μ m. (D) Blood glucose levels in control and *Sucnr1-βKO* mice in fasted or random-fed conditions (n=8). (E) Plasma insulin levels in control and *Sucnr1-βKO* mice in fasted or random-fed conditions (n=7-8). (F) Intraperitoneal (i.p.) and oral glucose tolerance tests in control and *Sucnr1-βKO* mice (n=6-8). Displayed are the blood glucose levels, area under the curve (AUC) and plasma insulin (n=5-6). (G) GLP-1 levels (n=5) during the glucose tolerance tests. (H) Insulin tolerance test in control and *Sucnr1-βKO* mice (n=6-8). (I) HOMA-IR index for control and *Sucnr1-βKO* mice (n=4-5). Data are presented as mean $\pm$ SEM or as box-

and-whiskers plots indicating the median, first and third quartiles, and maximum and minimum values. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs control mice, or when comparing experimental groups in orally-administered mice; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ when comparing experimental groups in i.p.-administered mice (Student's t -test in (E) and (I) when comparing two groups or two-way ANOVA with Bonferroni's test for multiple comparisons in (C-D) and (F-H)).

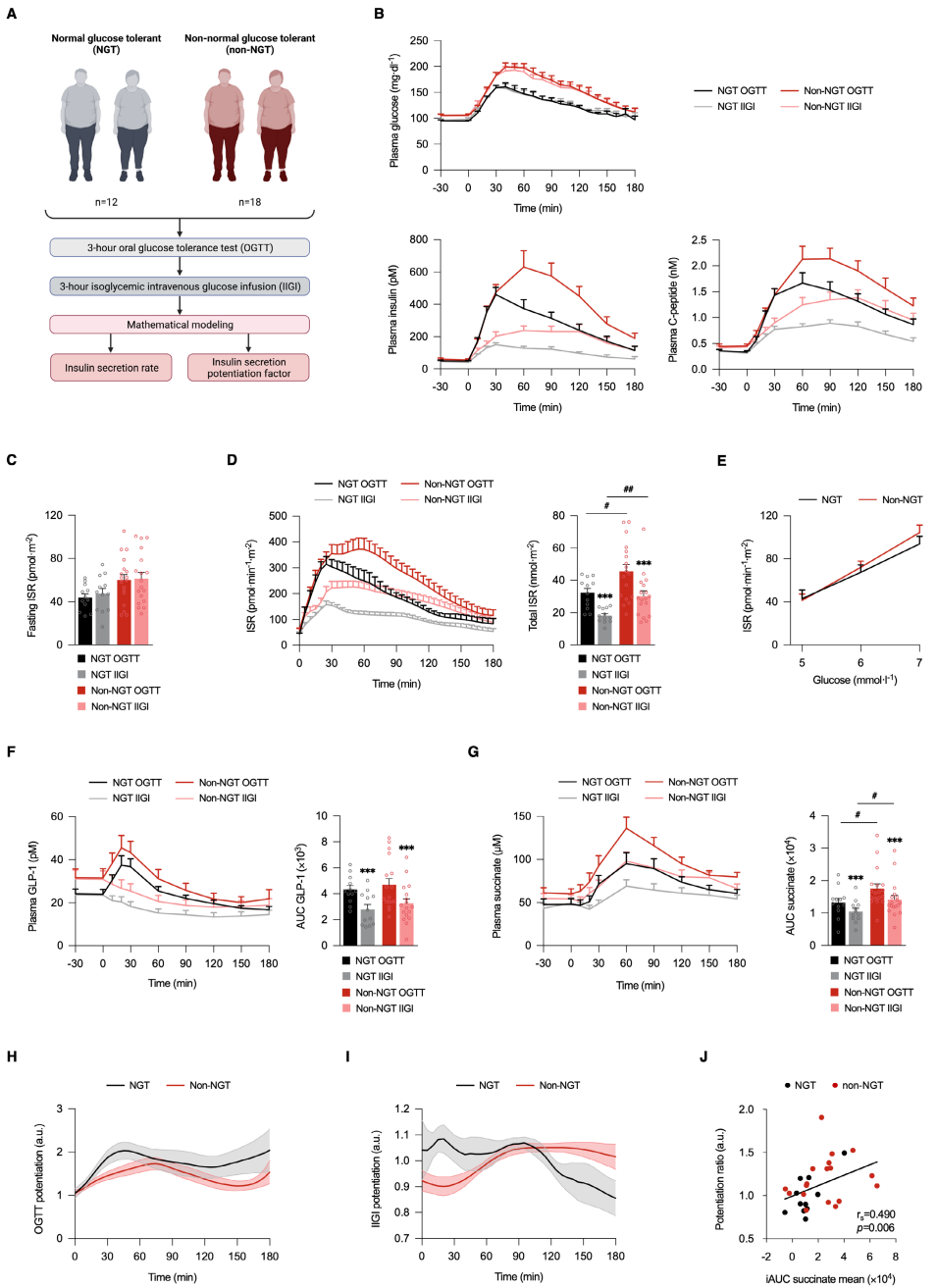


Figure 6: Succinate response is heightened in non-normal glucose tolerant patients and is associated with the potentiation of insulin secretion. (A) Study cohort and study schematic. (B) Plasma glucose, insulin, and C-peptide levels during the oral glucose tolerance test (OGTT) and isoglycemic intravenous glucose infusion (IIGI) in normal glucose tolerant (NGT) (n=12) and non-normal glucose tolerant (non-NGT) (n=18) patients. (C) Fasting insulin secretion rate (ISR) in NGT and non-NGT groups. (D) Total ISR during OGTT and IIGI tests in NGT and non-NGT groups. (E) β -cell glucose sensitivity represented as a dose-response function between ISR and glucose concentrations for both groups. (F) Plasma GLP-1 levels during OGTT and IIGI for both groups. (G) Plasma succinate levels during OGTT and IIGI for both groups. (H) Incretin-related potentiation calculated during OGTT in relation to IIGI for both groups. (I) Potentiation calculated during IIGI for both groups. (J) Correlation between the potentiation factor mean and the area under the curve (AUC) of succinate during the IIGI for both groups (n=30). Data are presented as mean $\pm$ SEM. * p <0.05, ** p <0.01, * p <0.001 when comparing OGTT and IIGI; * p <0.05, ** p <0.01, when comparing NGT and non-NGT subjects (paired and unpaired Student's t -tests in (C), (D), (F), and (G) Mann-Whitney U -test in (D), (F), and (G) Wilcoxon test in (D) (F) and (G) or two-way ANOVA tests in (E-I) or Spearman's rank correlation coefficient in (J).**

SUPPLEMENTAL INFORMATION

Supplemental Table 1. Characteristics of the islet donors (related to Figures 2B and 2D)

	Donor ID	Gender	Age (years)	BMI (kg·m ⁻²)	HbA1c (%)	Islet Purity (%)
Healthy	94/14	Female	32	21.8	n/a	90
	100/15	Male	41	24.7	n/a	80
	101/15	Female	51	22.4	n/a	90
	106/15	Male	80	20.8	n/a	85
	109/16	Female	61	20.6	5.2	80
	119/17	Male	51	29.4	n/a	85
	134/19	Male	48	29.3	4.8	90
Obese	97/14	Male	54	35.8	n/a	95
	104/15	Male	62	31.3	5.4	85
	110/16	Male	71	30.5	5.2	85

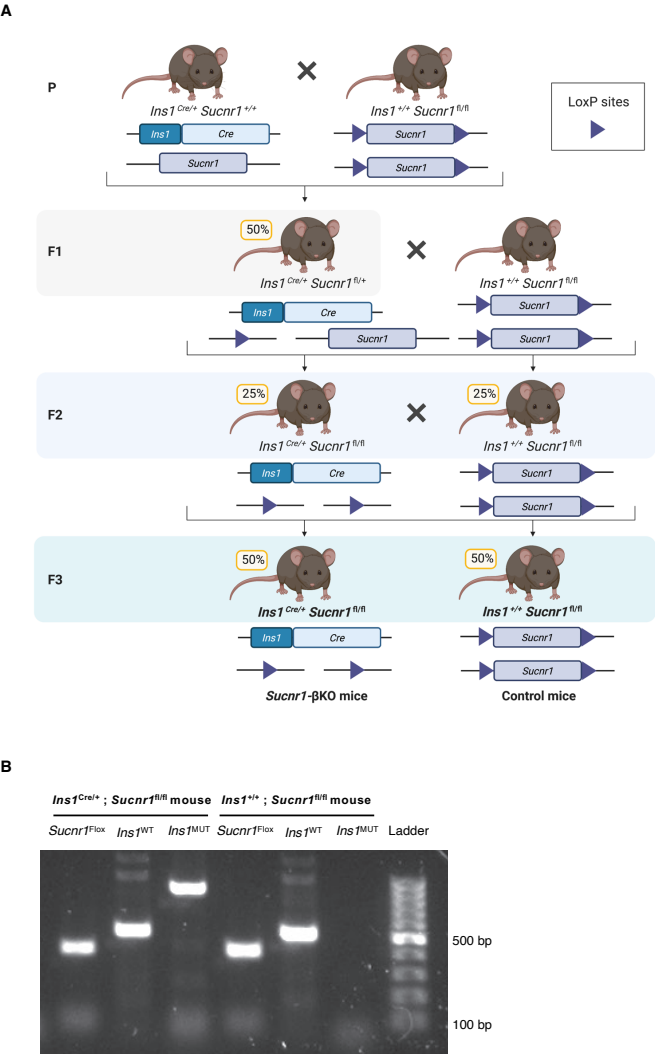
Abbreviations: Body mass index (BMI), hemoglobin A1c (HbA1c).

Supplemental Table 2. Characteristics of the islet donors (related to Figures 2C and 2D)

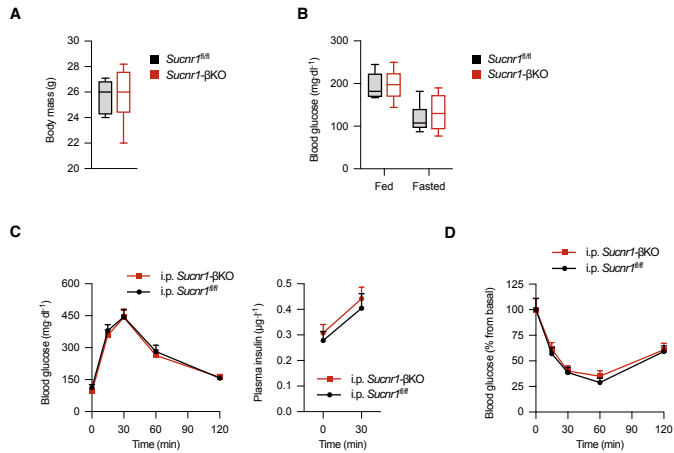
	Donor ID	Gender	Age (years)	Ethnicity	BMI (kg·m ⁻²)	HbA1c (%)	Purity (%)	Viability (%)
Healthy	HP-17031-01	Female	64	Hispanic/Latino	24.6	5.4	95	95
	UNOS ADJV268	Male	39	Caucasian	23.7	n/a	90	90
	UNOS ADIY035	Male	15	Hispanic/Latino	24.6	n/a	80	97
	HP-17036-01	Male	59	Caucasian	26.9	5.8	90	90
	HP-20199-01	Male	21	Hispanic/Latino	27.3	5.1	90	95
	MGH (n/a)	Male	38	n/a	28.3	n/a	90	90
Obese	RRID 12292085	Male	20	Hispanic/Latino	35.8	n/a	95	95
	UNOS ACEY097	Male	34	Caucasian	31.2	5.1	85	85
	HP-20206-01	Male	22	Hispanic/Latino	31.9	5.4	85	95
Type 2 Diabetes	UNOS ADHF167	Female	52	Hispanic/Latino	39.9	7.4	95	95
	HP-19078-T2D	Female	66	Caucasian	30.3	6.5	85	95
	HP-19171-T2D	Female	66	Hispanic/Latino	29.2	7.2	85	95
	RRID 12597653	Female	42	Hispanic/Latino	40.3	9.5	80	80
	HP-19131-T2D	Male	58	Caucasian	32.7	6.7	85	95
	HP-18320-T2D	Male	61	African American	27.4	7.1	85	95
	UNOS ADAHX156	Male	57	Hispanic/Latino	34.6	10.4	98	80

Abbreviations: Body mass index (BMI), hemoglobin A1c (HbA1c).

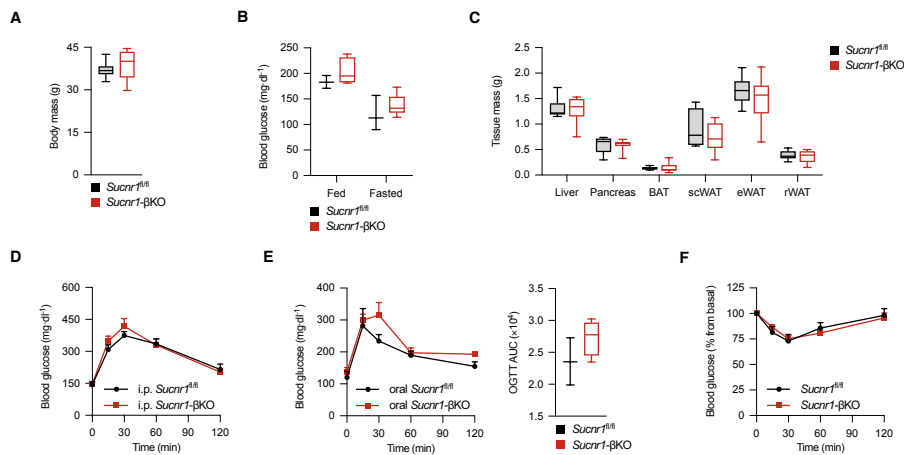
Annex



Supplemental Figure 1. Generation of β -cell-specific *Sucnr1* knockout mice and genotyping. (A) Breeding strategy to generate both control (*Ins1^{+/-};Sucnr1^{fl/fl}*) and *Sucnr1*- β KO (*Ins1^{Cre/+};Sucnr1^{fl/fl}*) mouse mice for the present study. (B) Genotyping of control and *Sucnr1*- β KO mice (*Sucnr1* floxed allele 450 bp; *Ins1* wild-type allele 524 bp; *Ins1* mutated allele 865 bp).



Supplemental Figure 2. *Sucnr1-βKO* mice under normal-chow diet (NCD). (A) Body mass of control and *Sucnr1-βKO* mice under NCD for 20 weeks (n=8-10). (B) Blood glucose levels in control and *Sucnr1-βKO* mice in fasted or random-fed conditions. (C) Intraperitoneal glucose tolerance test in control and *Sucnr1-βKO* mice; displayed are the blood glucose (n=8-10) and plasma insulin levels (n=6-7) during the test. (D) Insulin tolerance test in control and *Sucnr1-βKO* mice (n=8-10). Data are displayed as mean ± SEM.



Supplemental Figure 3. Old-age *Sucnr1-βKO* mice under normal-chow diet (NCD). (A) Body mass of control and *Sucnr1-βKO* mice under NCD at 54 weeks (n=7-8). (B) Blood glucose levels in control and *Sucnr1-βKO* mice in fasted or random-fed conditions (n=3-4). (C) Tissue mass at the end of the study at 54w (n=7-9). Brown adipose tissue (BAT), epididymal white adipose tissue (eWAT), and retroperitoneal white adipose tissue (rWAT). (D) Intraperitoneal glucose tolerance test in control and *Sucnr1-βKO* mice (n=7-9). (E) Oral glucose tolerance test in control and *Sucnr1-βKO* mice (n=3-4). (F) Insulin tolerance test in control and *Sucnr1-βKO* mice. Data are displayed as mean ± SEM.

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