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The role of cocaine- and amphetamine-regulated transcript in endocrine cells

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Type 2 diabetes (T2D) is a metabolic disease characterized by insufficient insulin secretion and insulin resistance. Disturbed beta-cell function is the final culprit that leads to the development of T2D. In addition, impaired incretin effect is likely a part of the pathogenesis of T2D. The aim of this thesis was to study the role of CART in beta-cells and incretin producing L- and K-cells.

Endogenous beta-cell CART was found to regulate insulin secretion and production, ATP levels and beta-cell exocytosis in INS-1 (832/13) cells. CART silencing (KD) decreased expression of genes important for exocytosis, glucose sensing, and insulin processing, likely as a consequence of reduced expression of beta-cell transcription factors *Mafa*, *Pdx-1*, *Isl1*, *NeuroD1*, *Nkx2.2* and *Nkx6.1*. Moreover, in human islets, *CARTPT* expression correlated with insulin, exocytosis genes and beta-cell transcription factors. To increase our understanding of the function of CART in beta-cells, we performed RNAseq on CART-silenced INS-1 (832/13) cells. Differential expression analysis revealed that CART KD affected expression of 25.5% of all detected genes, including genes with important roles for insulin secretion and exocytosis. The most strongly upregulated and downregulated genes after CART KD were *AABR07068253.1*, *Jun, Srp14* and *Adam11*, *Pak3*, *Ppp1r17* respectively. *Stxa1*, *Gnas* and *Stxbp1* were the top differentially expressed genes related to insulin secretion. In a follow-up study, we established the role of one of the strongest CART-regulated genes, SCRT1 in beta-cell function. SCRT1 expression was found in rodent and human beta-cells. SCRT1 was translocated from the nucleus to the cytosol in diabetic DEX rats and *SCRT1* mRNA expression was reduced by increasing glucose concentrations *in vitro*. SCRT1 KD in INS-1 (832/13) cells resulted in decreased insulin expression and secretion, as well as reduced expression of *Tcf7l2*, *Pdx-1*, *Isl1*, *Neurod1* and *Mafa*. In human islets, *SCRT1* expression correlated with insulin, glucagon, beta-cell transcription factors and exocytotic genes. Finally, we established CART expression in K- and L-cells in the human duodenum and jejunum and CART plasma levels were increased after a meal in humans. In incretin-producing GLUTag and STC-1 cells, CART mRNA was increased by fatty acids and GIP. In addition, CART KD in GLUTag cells reduced GLP-1 expression and secretion. CART also increased GLP-1 and GIP secretion during an oral glucose-tolerance test *in vivo* in mice. Collectively, our data imply that CART is an important regulator of insulin and incretin-producing cell function and glucose homeostasis. Thus, the therapeutic potential of CART-based therapy for treatment of T2D should be evaluated.

Key words: Type 2 Diabetes, T2D, cocaine and amphetamine-regulated transcript,CART, beta-cell, K-cell, L-cell, insulin, GIP, GLP-1, incretin hormones

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Cover photo by Liliya Shcherbina
The author's lab bench with cell culture plates creating an exponential curve. Symbolizes the development of the laboratory and scientific skills with time spent as a PhD student as well as countless hours spent in the cell lab.

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To my family

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1. **Shcherbina L**, Edlund A, Esguerra JL, Abels M, Zhou Y, Ottosson-Laakso E, Wollheim CB, Hansson O, Eliasson L, Wierup N. **Endogenous beta-cell CART regulates insulin secretion and transcription of beta-cell genes**. *Molecular and cellular endocrinology* 2017, **447**:52-60.
2. **Shcherbina L**, Reimegård J, Lindqvist A, Wierup N. **Characterization of genetic networks regulated by CART in INS-1 (832/13) cells.** *Manuscript*.
3. Chriett S, **Shcherbina L**, Edlund A, Lindqvist A, Abels M, Eliasson L, Wierup N. **The Transcriptional Repressor SCRT1 is a novel regulator of beta cell function.** *Manuscript.*
4. **Shcherbina L**, Lindqvist A, Thoren Fischer A-H, Ahlqvist E, Zhang E, Falkmer SE, Renström E, Koffert J, Honka H, Wierup N. **Intestinal CART is a regulator of GIP and GLP-1 secretion and expression.** *Submitted Manuscript.*

Papers not included in the thesis

1. Landerholm K, **Shcherbina L**, Falkmer SE, Jarhult J, Wierup N: Expression of cocaine- and amphetamine-regulated transcript is associated with worse survival in small bowel carcinoid tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2012, 18(13):3668-3676.
2. Banke E, Riva M, **Shcherbina L**, Wierup N, Degerman E: Cocaine- and amphetamine-regulated transcript is expressed in adipocytes and regulate lipid- and glucose homeostasis. *Regulatory peptides* 2013, 182:35-40.
3. Semenas J, Hedblom A, Miftakhova RR, Sarwar M, Larsson R, **Shcherbina L**, Johansson ME, Harkonen P, Sterner O, Persson JL: The role of PI3K/AKT-related PIP5K1alpha and the discovery of its selective inhibitor for treatment of advanced prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* 2014.
4. Zhou Y, Park SY, Su J, Bailey K, Ottosson-Laakso E, **Shcherbina L**, Oskolkov N, Zhang E, Thevenin T, Fadista J *et al*: TCF7L2 is a master regulator of insulin production and processing. *Human molecular genetics* 2014.
5. Abels M, Riva M, Bennet H, Ahlqvist E, Dyachok O, Nagaraj V, **Shcherbina L**, Fred RG, Poon W, Sörhede-Winzell M *et al*: **CART is overexpressed in human type 2 diabetic islets and inhibits glucagon secretion and increases insulin secretion**. *Diabetologia* 2016, **59**(9):1928-1937.
6. Zhou Y, Oskolkov N, **Shcherbina L**, Ratti J, Kock KH, Su J, Martin B, Oskolkova MZ, Göransson O, Bacon J *et al*: **HMGB1 binds to the rs7903146 locus in TCF7L2 in human pancreatic islets**. *Molecular and cellular endocrinology* 2016.

7. Tuomi T, Nagorny CLF, Singh P, Bennet H, Yu Q, Alenkvist I, Isomaa B, Östman B, Söderström J, Pesonen AK, Martikainen S, Räikkönen K, Forsén T, Hakaste L, Almgren P, Storm P, Asplund O, **Shcherbina L**, Fex M, Fadista J, Tengholm A, Wierup N, Groop L, Mulder H. **Increased melatonin signaling is a risk factor for Type 2 Diabetes.** *Cell Metabolism*. 2016;23(6): 1067-77.

8. Lindqvist A, **Shcherbina L**, Thorén Fischer AH, Wierup N. **Ghrelin is a regulator of GLP-1 secretion and transcription in mice.** *Frontiers in Endocrinology.* 2017 (in press)

9. Sjögren M, Duarte AI, McCourt AC, **Shcherbina L**, Wierup N, Björkqvist M. **Ghrelin rescues skeletal muscle catabolic profile in the R6/2 mouse model of Huntington´s disease.** *Scientific reports.* 2017 (in press).

Abbreviations

Alpha-KIC	alpha-ketoisocaproate
Arc	arcuate nucleus
ATP	Adenosine triphosphate
cAMP	cyclic AMP
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
Cpe	carboxypeptidase E
CRE	cAMP responsive element
CREB	CRE-binding protein
DEX	dexamethasone
DM	diabetes mellitus
DPP4	dipeptidyl peptidase-4
EPAC2	exchange protein directly activated by cAMP 2
ER	endoplasmic reticulum
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
GAD	glutamic acid decarboxylase
GCK	glucokinase
GI	gastrointestinal
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
GLUT	glucose transporter
GPCR	G protein-coupled receptor
GSIS	glucose-stimulated insulin secretion
GWAS	genome-wide association studies
HbA1c	haemoglobin A1c
IAPP	islet amyloid polypeptide
IBMX	isobutylmethylxanthine
ICV	intracerebroventricular
IP3	inositol 1, 4, 5-triphosphate
ISL1	islet-1
K^+_{ATP} channel	ATP-sensitive potassium channel

LADA	latent autoimmune diabetes in adults
LHA	lateral hypothalamus area
MAFA	MAF BZIP Transcription Factor A
MAPK	mitogen-activated protein kinase
MMT	mixed-meal test
MODY	maturity onset diabetes in the young
NAc	nucleus accumbens
NEUROD1	Neuronal Differentiation 1
NKX	NK2 Homeobox
NPY	neuropeptide Y
NRSE	neuron-restrictive silencer element
NRSF	neuron-restrictive silencer factor
OGTT	oral glucose tolerance test
PDX-1	pancreatic and duodenal homeobox 1
PKA	protein kinase A
PKC	protein kinase C
PP	Pancreatic polypeptide
PVN	paraventricular nucleus
PYY	peptide YY
RP	releasable pool
RRP	ready releasable pool
SCRT1	scratch family transcriptional repressor 1
SNAP25	synaptosomal-associated protein 25kDa
SNARE	soluble NSF attachment protein receptor
SST	somatostatin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TCF7L2	transcription factor 7-like 2

Summary

Type 2 diabetes (T2D) is a metabolic disease characterized by insufficient insulin secretion and insulin resistance. Disturbed beta-cell function is the final culprit that leads to the development of T2D. In addition, impaired incretin effect is likely a part of the pathogenesis of T2D. The aim of this thesis was to study the role of CART in beta-cells and incretin producing L- and K-cells.

Endogenous beta-cell CART was found to regulate insulin secretion and production, ATP levels and beta-cell exocytosis in INS-1 (832/13) cells. CART silencing (KD) decreased expression of genes important for exocytosis, glucose sensing, and insulin processing, likely as a consequence of reduced expression of beta-cell transcription factors *Mafa*, *Pdx-1*, *Isl1*, *NeuroD1*, *Nkx2.2* and *Nkx6.1*. Moreover, in human islets, *CARTPT* expression correlated with insulin, exocytosis genes and beta-cell transcription factors. To increase our understanding of the function of CART in beta-cells, we performed RNAseq on CART-silenced INS-1 (832/13) cells. Differential expression analysis revealed that CART KD affected expression of 25.5% of all detected genes, including genes with important roles for insulin secretion and exocytosis. The most strongly upregulated and downregulated genes after CART KD were *AABR07068253.1*, *Jun, Srp14* and *Adam11*, *Pak3*, *Ppp1r17* respectively. *Stxa1*, *Gnas* and *Stxbp1* were the top differentially expressed genes related to insulin secretion. In a follow-up study, we established the role of one of the strongest CART-regulated genes, SCRT1 in beta-cell function. SCRT1 expression was found in rodent and human beta-cells. SCRT1 was translocated from the nucleus to the cytosol in diabetic dexamethasone (DEX)-treated rats and *SCRT1* mRNA expression was reduced by increasing glucose concentrations *in vitro*. SCRT1 KD in INS-1 (832/13) cells resulted in decreased insulin expression and secretion, as well as reduced expression of *Tcf7l2*, *Pdx-1*, *Isl1*, *Neurod1* and *Mafa*. In human islets, *SCRT1* expression correlated with insulin, glucagon, beta-cell transcription factors and exocytotic genes. Finally, we established CART expression in K- and L-cells in the human duodenum and jejunum and CART plasma levels were increased after a meal in humans. In incretin-

producing GLUTag and STC-1 cells, CART mRNA was increased by fatty acids and GIP. In addition, CART KD in GLUTag cells reduced GLP-1 expression and secretion. CART also increased GLP-1 and GIP secretion during an oral glucose-tolerance test *in vivo* in mice. Collectively, our data imply that CART is an important regulator of insulin and incretin-producing cell function and glucose homeostasis. Thus, the therapeutic potential of CART-based therapy for treatment of T2D should be evaluated.

Populärvetenskaplig sammanfattning

Typ 2 Diabetes (T2D) utgör ett globalt hälsoproblem som orsakar stora kostnader för samhället och lidande för patienterna. I världen finns idag över 280 miljoner diabetespatienter och var sjätte sekund dör en person till följd av diabetes eller diabeteskomplikationer. I Sverige finns idag över fyrahundratusen patienter med diabetes och antalet ökar stadigt.
T2D kännetecknas av förhöjda blodsockernivåer på grund otillräcklig produktion av insulin från de Langerhanska öarna i bukspottkörteln i kombination med att insulinets målceller utvecklar minskad känslighet för insulin. Dessutom fungerar inte inkretinhormonerna GIP och GLP-1, som ökar insulinutsöndring efter måltid, på ett korrekt sätt. I denna avhandling beskrivs hur hormonet CART reglerar produktion och utsöndring av insulin, GIP och GLP-1.
CART är ett hormon som stimulerar insulinutsöndring och främjar överlevnad av insulinproducerande betaceller. Produktionen av CART är ökad i de insulinproducerande betacellerna från T2D patienter och hos diabetiska råttor och möss.

Denna avhandling är indelad i fyra delar. De två första beskriver betydelsen av CART för betacellernas normala funktion. Detta testades genom att reducera CARTs genuttryck med siRNA. Vi upptäckte att CART verkar för att öka utsöndring och produktion av insulin, sannolikt genom att reglera genuttrycket av ett antal viktiga transkriptionsfaktorer och exocytosproteiner. Vi kunde även påvisa att genuttrycket av CART korrelerar med insulin och andra gener som är viktiga för normal betacellsfunktion i RNA-sekvenseringsdata från öar från 195 organdonatorer. Detta talar för att CART har betydelse även för funktionen av människans betaceller.

För att få djupare förståelse för de exakta mekanismerna bakom CARTs effekt på insulinproduktion och utsöndring analyserades betaceller med reducerat CART genuttryck med hjälp av RNA-sekvensering. Hämning av CARTs genuttryck visade sig påverka 25% av alla påvisade gener i cellen. Flera av de kraftigast reglerade generna har betydelse för

insulinproduktion och frisättning, samt cellöverlevnad. Nätverksanalyser av de påverkade generna föreslår helt nya mekanismer för CARTs effekt på framför allt insulin sekretion.

I den tredje delen av avhandlingen studerades betydelsen av en av de mest CART-reglerade generna, SCRT1 i betaceller. SCRT1 är en transkriptionsfaktor med hittills okänd funktion i betaceller. Produktion av SCRT1 kunde påvisas i insulinproducerande celler i bukspottkörteln hos människor, möss och råttor. En koppling mellan T2D och SCRT1 kunde konstateras genom att lokalisering av SCRT1 i betaceller skiljde sig mellan diabetiska och friska råttor. Detta talar för minskad SCRT1-aktivitet vid T2D. När genuttrycket för SCRT1 hämmades i betaceller minskade både utsöndring och produktion av insulin, samt genuttrycket för viktiga betacellgener. Slutligen korrelerade genuttrycket av SCRT1 med insulin, glukagon och andra gener med viktiga funktioner i betaceller i RNA-sekvenseringsdata från humana öar.

I den fjärde delen av avhandlingen studerades funktionen av CART i inkretinproducerande celler. CART upptäcktes vara en ny beståndsdel i GIP-producerande K-celler och GLP-1-producerande L-celler i human tunntarm. Dessutom ökade koncentrationen av CART i blodet efter en måltid i friska försökspersoner. Hämmat genuttryck av CART i GLP-1-producerande celler orsakade minskad utsöndring och produktion av GLP-1. I GIP-producerande celler visades å andra sidan hämmande effekter av CART på GIP produktion. Slutligen visade sig intravenös administration av CART leda till ökad GLP-1 och GIP utsöndring i levande möss.
Sammantaget har jag i denna avhandling visat att CART har en viktig roll i insulin- och inkretinproducerande celler. Resultaten talar för att CART har egenskaper som gör CART-baserade ämnen intressanta för utveckling av nya behandlingar mot T2D.

Introduction

Diabetes

Diabetes mellitus (DM) is a disorder of chronically elevated circulating blood glucose levels. According to the International Diabetes Federation, diabetes affects more than 280 million people worldwide, and every six seconds a person dies from diabetes or diabetes-related complications. Although the prevalence of diabetes in Sweden (4.7%) is lower than worldwide (8.8%), currently almost 447 thousand people are estimated to have diabetes within the nation. In USA 29.3 million people (11.3% of the population) are affected by diabetes. India and China have the highest prevalence of diabetes with 69.2 million (9.3% of the population) and 109 million (12.1% of the population) people affected in 2015 respectively. Diabetes leads to a number of complications, some of which are associated with increased mortality and diabetes is a leading cause of cardiovascular disease, blindness, kidney failure and lower limb amputation in high income countries [1].

Diabetes is diagnosed by fasting plasma glucose levels ≥ 7 mmol/l (126 mg/dl), by a plasma glucose concentration ≥ 11.1 mmol/l (200 mg/dl) 2 h after a 75g oral glucose load or haemoglobin A1c (HbA1c) levels of above 6.5% (48 mmol/mol). HbA1c is a glycosylated form of haemoglobin, representing the average blood glucose levels during the last eight to twelve weeks [2].

Diabetes has traditionally been divided into Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D). T1D is characterized by autoimmune destruction of pancreatic beta-cells leading to diminished insulin secretion. Most patients with T1D have autoantibodies against e.g. glutamic acid decarboxylase (GAD), insulin, or zinc transporter 8. T1D is mainly diagnosed in children and adults under the age of 35 years [3]. T2D, on the other hand, is characterized by a combination of insulin resistance and dysregulated insulin secretion. T2D patients often initially hyper secrete insulin as a compensation for the increased demand for insulin. In later stages of the disease the beta-cells are no longer able to compensate and

fulminant T2D develops. T2D is often associated with higher BMI and higher age, although it is increasingly reported in adolescents in India and China [3].
However, this classification poorly describes the true range of diabetes with several different overlapping mechanisms leading to the disease [4]. Rather than being dichotomously distinct disorders, T1D and T2D can be considered as the two ends of a diabetes spectrum, with the intermediates being maturity onset diabetes of the young (MODY), latent autoimmune diabetes in adults (LADA) and other subtypes [3].
The current classification of diabetes is mostly based on age of onset, along with the presence of either obesity and metabolic syndrome or insulin deficiency and autoantibodies. None of these criteria are clear-cut and classification can in some cases be difficult [4]. The All New Diabetics in Scania (ANDIS) project in southern Sweden represents a new initiative to reclassify diabetes into subgroups with the aim of improving diagnosis and treatment strategies [5]. At the time of registration, blood samples are drawn to determine the presence of GAD antibodies, levels of C-peptide, fasting glucose, and HbA1c. These data have been used to classify the disease into five subtypes leading to a novel diabetes classification [3]. Stratification of patients into different subgroups is an important step towards precision medicine, guiding the choice of treatment and ability to predict disease progression, treatment outcome and risk of diabetic complications.

T2D risk factors

T2D is a complex disease resulting from an interaction between genes and environment. Risk factors for T2D include age, sex, obesity, low physical activity, diet, smoking, ethnicity, family history of T2D, history of gestational diabetes mellitus and different drug treatments [6-11]. The contribution of genetics to T2D risk has been shown through family-based studies and twin studies [12, 13]. The heritability of T2D has been estimated to be 25-80% with the highest estimates in the studies with longer follow-up periods [3]. The risk of T2D development is 40% for individuals with one parent having T2D and nearly 70% for individuals with both parents having T2D. [3]. Notably, the risk of T2D is greater if the mother has T2D. This is opposite to the situation in T1D, where the risk is greater if the father is affected [12, 14]. Multiple genes are thought to be involved, each producing a small effect on T2D risk [15]. Advances in technology and analytical approaches have led to the progress in the identification of genetic variants contributing to T2D susceptibility. Using

a candidate-gene approach, *PPARG* was the first gene identified [16]. Since 2007, genome wide association studies (GWAS) have identified more than 150 genetic variants mapping to >120 loci. *TCF7L2* harbors common genetic variants with the strongest effect on T2D risk (SNP rs7903146) [17-19]. However, the >80 loci identified explain less than 20% of the heritability of T2D [3, 10, 20, 21]. Possible explanations for the missing heritability include unidentified common variants, distorted parent-of-origin transmission of risk alleles, rare variants, and gene–gene and gene–environmental interactions in which epigenetic effects might be important [3, 22].

A rather new concept in T2D heritability research is that changes in the epigenome may contribute to the increased risk of T2D. Environmental factors are indeed important for T2D development and epigenetic modifications may mediate the effects of environment on gene expression. Interestingly, islets from T2D donors display altered DNA methylation [23, 24]. Additionally, altered methylation of several genes paralleled by significant changes in gene expression was observed in T2D islets and was linked to beta-cell dysfunction in *in vitro* studies [25]. An experimental study provides further support by showing that palmitate treatment of human islets was linked to altered methylation, gene expression and decreased insulin secretion [26].

Thus, despite recent advances in the field of genetics and identification of numerous genetic factors associated with T2D, we have only explained a small part of the total T2D heritability.

The pancreas

The pancreas is a glandular organ located in the abdominal cavity that consists of exocrine and endocrine tissue. The exocrine compartment is made up of acinar cells that produce and secrete digestive enzymes, water and pH-neutralizing bicarbonate via a duct system into the duodenum. The endocrine tissue comprises only 2-3% of the total pancreas mass and is composed of scattered cell clusters known as the islets of Langerhans. The islets of Langerhans were named by G. E. Laguesse in 1893 [27] in honor of Paul Langerhans who made the discovery in 1869 [28].

The pancreatic islets

The islets are highly vascularized micro-organs that receive a disproportionately large fraction of the pancreatic blood flow (up to 5-10% of total pancreatic blood flow) compared with the exocrine portion of the pancreas. In addition, islet blood supply regulation is independent from

that of the exocrine pancreas [29-31]. Blood vessels in the islet vascular network are wider and more numerous compared to the vessels of the surrounding exocrine tissue [32]. Endothelial cells in the capillaries supplying blood to endocrine cells contain more fenestrae than the exocrine pancreatic capillaries, illustrating the close interaction between the islet cells and the circulation which is important for hormone passage into the circulation [31, 33]. The islets range in size from just a few cells to clusters of several thousands of cells with a diameter of 300-400 μm [34]. The islets consist of several cell types that function together to maintain glucose homeostasis. The major hormone-secreting cell types are beta-cells (insulin), alpha-cells (glucagon) [35, 36], delta-cells (somatostatin (SST)) [35, 37], PP-cells (pancreatic polypeptide) [38, 39] and epsilon-cells (ghrelin) [40, 41]. In addition to the classical islet hormones, the islet cells express a number of neurohormonal peptides, including islet amyloid polypeptide (IAPP), peptide YY (PYY), neuropeptide Y (NPY) [42], apelin [43], nesfatin-1 [44] and CART [45]. Recently, glucose-dependent insulinotropic polypeptide (GIP) [46] and glucagon-like peptide-1 (GLP-1) [47-49] production in the alpha-cells was also reported.

The pancreatic islets display diverse patterns of endocrine cell arrangement in different species. Most mammals have a mantle islet structure with beta-cells clustered in a central core surrounded by smaller numbers of alpha-cells, delta-cells, PP-cells and epsilon-cells in the periphery [34, 35]. Mouse and rat islets contain 60-80% beta-cells in the core of the islet surrounded by a mantle of around 20% alpha-cells, less than 10% delta-cells, 1% PP-cells and rare ghrelin cells in adult animals. Human islets have a higher percentage of alpha-cells (60% beta-cells and 30% alpha-cells, 10% delta-cells, 1% ghrelin-cells) and a more scattered organization of the different endocrine cell types [41, 50]. Thus, beta-cells and non-beta-cells are dispersed throughout the islet [50] and this organization likely predisposes human islets to strong paracrine interactions and facilitates the fine-tuning of islet hormone release [51].

Islet cell signatures and expression changes in T2D

Recognition of impaired beta-cell function, in addition to insulin resistance, as a key problem in T2D has been one of the main changes in the field in the past decade. Beta-cell failure and beta-cell death, and reduced beta-cell mass are no longer considered secondary to insulin resistance. In addition, growing evidence suggests that the beta-cells lose insulin expression rather than die, possibly due to dedifferentiation

towards a progenitor state, and subsequent conversion into other islet cell types [52, 53].

A number of studies have suggested that beta-cell dysfunction in T2D includes a loss of cell identity. This is characterized by reduced expression of proteins associated with mature beta-cell function e.g. insulin and the transcription factors MAFA, PDX1, NKX6.1, and by dedifferentiation, regression to a progenitor or stem cell-like state (reviewed in [53]). Importantly, the differentiation status of the beta-cells is not only dependent on the presence of certain proteins, but also requires suppression of so called disallowed genes, genes that are selectively repressed in beta-cells in order to prevent inappropriate beta-cell responses e.g. insulin release triggered by exercise [54, 55]. The disallowed genes include monocarboxylate transporter-1 (MCT-1), lactate dehydrogenase (LDHA), hexokinase (HK) and repressor element 1 silencing transcription factor (REST) [54, 56].

RNA-sequencing of human islets from control and T2D donors has been used to identify genes of potential importance in the pathogenesis of T2D [57]. Recently, single-cell RNA sequencing has emerged as a powerful tool used for identification and characterization of cell populations, cellular states, transcriptional profiles and alterations in gene expression in among others cancer [58], neuroscience [59, 60] and diabetes research [61-63]. In the paper by Baron *et al.*, the authors identified 15 subpopulations of human pancreatic cells including alpha-, beta-, gamma-, delta-, and epsilon-cells, acinar, ductal, quiescent and activated pancreatic stellate cells, vascular cells, immune cell types and Schwann cells. Interestingly, the authors reported heterogeneity within beta-cells in the regulation of genes related to functional maturation and levels of endoplasmic reticulum (ER) stress [61]. Segerstolpe *et al*. uncovered subpopulations of alpha-, beta- and acinar cells. Interestingly, five sub-populations of beta-cells expressed *INS* at similar levels with combinatorial expression of *RBP4*, *FFAR4*/*GPR120*, *ID1*, *ID2* and *ID3* [62]. Notably, the authors observed alterations in gene expression in both exocrine and endocrine cell types of T2D islets and identified potential compensatory mechanisms for the decreased beta-cell mass in T2D, as downregulation of *FXYD2* and upregulation of *GPD2* [62]. In the report by Wang *et al.*, the authors showed that the beta-cell gene signatures of T2D samples were less defined, compared with healthy adults, and resembling those of less mature juvenile cells [63].

There is also growing evidence suggesting that islet cells can, in addition to dedifferentiating into progenitor-like cells, also trans-differentiate to

other islet cell types. Alpha-to-beta or beta-to-alpha conversions have been reported in cell-type-specific deficiency and in overexpression models for *Arx* [64, 65], *Pdx*-1 [66], *Pax4* [67] and *Dnmt1* [68]. In addition, *Mafa* has recently been shown to potentiate the ability of *Pdx-1* to induce beta-cell formation from *Ngn3*-positive endocrine precursors and from alpha-cells [69].

The reported beta- and islet cell heterogeneity and plasticity formed the rationale for a number of studies suggesting that beta-cell dedifferentiation is behind the beta-cell failure in T2D. Dedifferentiated beta-cells, characterized by upregulation of embryonic progenitor markers (*Ngn3*, *L-Myc*, *Nanog* or *Oct4*), were found under metabolically stressful conditions and in mouse T2D models [70-72]. Moreover, insulin treatment of the mice normalized glucose levels and led to re-differentiation of Ngn3-positive into insulin-positive beta-cells [71, 72], suggesting that dedifferentiation can be reversed. Thus, it is now a very exciting time for diabetes research when advances in technology lead to break-throughs in uncovering the mechanisms behind beta-cell failure in T2D.

Insulin

Insulin secreted from islet beta-cells plays a major role for the maintenance of energy homeostasis. Insulin secretion is tightly regulated to maintain blood glucose levels within a narrow range. Short-term regulation of insulin secretion e.g. in response to a meal occurs mainly at the level of exocytosis. However, the maintenance of adequate intracellular stores of insulin on a long-term basis relies on the transcriptional and translational regulation of insulin biosynthesis [73].

Insulin expression

Rodents have two non-allelic insulin genes *Ins1* and *Ins2,* while most other mammals have a single copy of the insulin gene [74]. *Ins2* is an ortholog to the insulin genes in the other mammals, and *Ins1* is a rodent-specific retrogene.

Tissue-specific expression and regulation of the insulin gene is mediated by the insulin promoter [75-77]. Many transcription factors act upon this region, binding within the insulin enhancer region (between nucleotides -340 and -91). All characterized insulin genes share conserved DNA motifs mediating regulation of transcription; E, A and C1/RIPE3b elements being major players [78].

E elements share the core sequence CANNTG. E-boxes bind dimeric

complexes of islet enriched (BETA2/NeuroD) and generally distributed basic helix-loop helix family proteins (bHLH) (E12/E47 and E2/5). NeuroD is expressed in pancreatic and neuroendocrine cells and regulates both insulin expression and pancreas development [79, 80] and *Beta2* KO mice have been reported to have decreased beta-cell number, impaired islet formation and to develop diabetes [80].

A elements contain AT rich sequences (A1-A5). All of them (except A2, containing the GGAAAT sequence) contain the core TAAT sequence, a binding site for homeodomain proteins. PDX-1, a major transactivator of the insulin gene [81-83], as well as islet specific genes, e.g. glucose transporter 2 (GLUT2) [84], glucokinase (GCK) [85, 86], IAPP [87] and somatostatin [88], binds to A-boxes. PDX-1 is crucial for pancreas development as *Pdx-1*-deficient mice [89] and humans with mutated *PDX-1* [90] demonstrate pancreas agenesis. Silencing of PDX-1 in pancreatic islets or clonal beta cells leads to impaired insulin secretion and expression [85]. In the rat *Ins1*, A3 and A4 boxes bind additional proteins with expression limited to few cell types. These additional proteins include ISL1, CDX3, LMX-1, HNF-1α. ISL1 is expressed in several islet cell types and is important for islet formation during development [91]. ISL1 silencing in beta-cells results in decreased insulin expression and secretion [85].

C1/RIPE3b element (-118/-107) binds two complexes, the beta cell-specific RIPE3b1 and the more generally distributed RIPE3b2. The MAFA transcription factor was identified as a binding partner for RIPE3b1. MAFA is a beta-cell specific transcription factor during development and in the adult pancreas [92] regulating insulin transcription and secretion, as well as glucose-regulated and fatty acid-inhibited insulin expression [93-95].

C2 element contains a CAGG repeat (in the human insulin gene, -253 to -244) and related CACC sequence (*Ins1* gene, -329 to -307). PAX4, a transcriptional repressor during early pancreas development, and PAX6, a transactivator, both bind to the C2 element [78, 96].

The close proximity of regulatory elements binding a number of transcription factors and co-regulators in the insulin promoter results in a variety of outcomes through additive and synergistic interactions between the bound proteins. In addition, regulatory elements can overlap and

thereby introduce another layer of complexity through binding competition between alternative transcription factors [97].

Thus, insulin transcription is controlled by sophisticated mechanisms reassuring beta-cell specific insulin expression and adequate response to various stimuli reflecting the important role of insulin in the regulation of metabolism.

Insulin secretion and regulation of blood glucose

Insulin is a 6 kDa hormone consisting of one A- and one B-polypeptide chain, linked together by two disulfide bonds. It is synthesized as preproinsulin, a single polypeptide, containing C-peptide (the central portion of proinsulin, connecting A and B chains) and a 24-residue signal peptide that directs preproinsulin to the rough ER (RER). Cleavage of the signal peptide and translocation of preproinsulin into the RER leads to the formation of proinsulin (establishing disulfide bonds and the folded conformation). As proinsulin is being transported through the Golgi apparatus, it undergoes maturation through the action of cellular endopeptidases prohormone convertases (PC1 and PC2) as well as the exoprotease carboxypeptidase E (CPE), leading to the cleavage of C-peptide, and the A and B chains. Insulin forms a complex with Zn^{2+} leading to formation of the dense-core of the granules. Of the proinsulin processed in the Golgi apparatus approximately 99% remains in storage in secretory granules for regulated insulin release. The remaining 1% of proinsulin escapes storage in granules whereby proinsulin maintains a low rate of constitutive insulin secretion [98].

Increased glucose levels lead to glucose transporter 2 (GLUT2)–mediated insulin-independent glucose uptake into the beta-cells. In human beta-cells this is achieved predominantly through GLUT1 [99]. The low substrate affinity of GLUT2 ensures high glucose influx into the cell. After entering the beta-cells, glucose is phosphorylated by glucokinase, the rate-limiting step in beta-cell glucose metabolism [100]. Glucokinase exhibits relatively low affinity for glucose with a K_m of 6 mmol/L and is not inhibited by its product, thereby enabling its continued activity despite a high glycolysis load [100]. Glucose metabolism leads to an increase in ATP production and increased ATP/ADP ratio, which in turn leads to the closure of the ATP-sensitive K^+_{ATP} channels and depolarization of the plasma membrane. This leads to the opening of voltage-sensitive Ca^{2+} channels (VDCC), and Ca^{2+} influx into the cell, which triggers exocytosis of insulin. This mechanism is referred to as the triggering pathway of insulin secretion [101].

However, insulin granules have to undergo docking and priming before the content can be released [102, 103]. Insulin granules that are docked at the plasma membrane form the so called readily releasable pool (RRP, 1-5% of all granules) and insulin granules in the cytoplasm form the reserve pool (RP). Furthermore, part of the RRP granules is docked and primed forming the immediate releasable pool (IRP) of insulin granules that are secreted during the first phase of insulin secretion. The priming of the remaining granules in the RRP is ATP-dependent and responsible for the second phase of insulin secretion [104, 105].

A number of proteins are directly responsible for coordinating exocytosis of insulin. The assembly of Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins has been shown to mediate docking and fusion of the insulin granules at the plasma membrane. [103]. The main SNARE proteins include granular vesicle associated membrane protein 2 (VAMP2)/synaptobrevin 2, as well as proteins associated with the plasma membrane e.g. syntaxin 1A (STX1A), synaptosomal-associated protein 25kDa (SNAP25), MUNC18 (or syntaxin binding protein; STXBP1) and Ca^{2+} sensing synaptotagmins [106]. SNAP25 and STX1A have been demonstrated to have a fundamental role in beta-cell exocytosis [107, 108] and STX1A is mainly known to be a positive regulator of beta-cell exocytosis [107] but has also been shown to act as a negative regulator of insulin release [109]. Moreover, STX1A together with STXBP1 are critical for the formation of granular docking sites [106] and STXBP1 together with SYTL4 are important for docking [110].

In addition to this triggering pathway, there is also an amplifying pathway of insulin secretion. Various hormones and neurotransmitters activate, via receptors on the beta-cell surface, pathways that potentiate nutrient-stimulated insulin secretion. For example, the incretin hormone GLP-1 potentiates insulin secretion via GLP1R, which will be described in detail in the incretin section below. The metabolic amplifying pathway is provoked by metabolites from glucose metabolism and accounts for about 50% of glucose-induced insulin secretion [111]. Messengers involved in the amplifying pathway can act directly on insulin release or through activation of mitochondrial signaling, including GTP, ATP, cAMP, NADPH and others [101, 104, 112]. Interestingly, recent advances suggest the possibility that the amplifying pathway contributes to both the first and the second phase of glucose-stimulated insulin secretion [111].

Insulin is released from the pancreas in a pulsatile manner with 4-6 min intervals in humans. Importantly, impairment in insulin oscillations is seen in T2D patients (and their close relatives). The pulsatile insulin secretion

from the pancreas requires coordination of secretory activity of individual islets mediated by e.g. intrapancreatic nerves and neuron-derived ATP, nitric oxide (NO) and carbon monoxide. Within the islet, insulin release is synchronized by oscillations in intracellular Ca^{2+} levels resulting from spreading of the depolarization by gap junctional coupling between beta-cells (reviewed [113]).

Insulin action and insulin resistance

Secreted insulin acts on a variety of tissues, including adipose tissue, muscle and liver through the activation of the insulin receptor (IR) and IR signaling. In adipose tissue and muscle cells, binding of insulin to IR leads to translocation of GLUT4 to the cell membrane and subsequently increased glucose uptake. Glucose can then be converted into triglycerides or glycogen for energy storage [114]. Hepatic glucose uptake is GLUT2-mediated and insulin-independent. In hepatocytes the activation of IR signaling leads to increased glycogen synthesis and inhibition of gluconeogenesis [115].

Insulin resistance is a hallmark of obesity and a cornerstone in T2D pathophysiology. In the insulin resistant state, the metabolic response of target cells, e.g. cells in liver, skeletal muscle and adipose tissue, to insulin is decreased. The consequence is decreased glucose uptake and insufficient suppression of endogenous glucose production in the liver, resulting in impaired blood glucose lowering ability of circulating or injected insulin [116, 117]. There are two opposing hypotheses on when and how insulin resistance is developed in obesity and T2D. According to the first one, over nutrition deregulates hepatocyte regulators of gluconeogenesis (e.g. FOXO1) leading to an increase in hepatic glucose output. In the muscle, the decreased GLUT4-mediated response to insulin results in decreased glucose uptake. These disruptions and decreased responsiveness of the adipose tissue to insulin lead to hyperglycemia and subsequent hyperinsulinemia, which in turn activates hepatic lipogenesis, leading to hyperlipidemia. The second hypothesis proposes that hyperinsulinemia develops first, as a result of overfeeding and obesity and that this in turn leads to insulin resistance in the target tissues via downregulation of insulin signaling, activation of inflammatory pathways and other indirect pathways (reviewed [116]).

Glucagon

Between meals, glucagon is released from the pancreatic alpha-cells to prevent glucose levels from falling. Glucagon achieves this mainly by

stimulating processes that lead to increased hepatic glucose output. Thus, glucagon promotes glycogenolysis, *i.e.* the breakdown of glycogen into glucose, and also inhibits glycogen synthesis. In addition, glucagon increases gluconeogenesis, *i.e.* the formation of new glucose molecules and decreases glycolysis in the liver [118]. Importantly, glucagon secretion is impaired in T2D with an excess of glucagon being secreted during hyperglycemia and insufficient glucagon being secreted to normalize hypoglycemia [119]. In the current view, impaired glucagon secretion contributes to the excessive hepatic glucose production seen in T2D, thereby exacerbating hyperglycemia [120].

GPCR-mediated regulation of insulin secretion

In addition to glucose, insulin and glucagon levels are regulated by other circulating factors, including free fatty acids, neurotransmitters and hormones with G protein-coupled receptors (GPCRs) mediating their effects [121].

G proteins consist of α, β, and γ protein subunits. In the inactive form the Gα subunit binds guanine diphosphate (GDP). Upon activation, the Gα subunit releases GDP and binds guanine triphosphate (GTP). The Gα subunit then releases the βγ complex, and the Gα subunit and the βγ complex each activate a variety of effector molecules. Each GPCR complex has unique Gα subunits from one of the four major classes of α subunits (G_s, G_i, G_q, and $G_{12/13}$), and each class consists of multiple subtypes. GPCRs signaling through the $G\alpha_q$ pathway activates phospholipase C (PLC) to generate diacylglycerol and inositol 1,4,5 trisphosphate, (IP_3) which mobilizes intracellular Ca^{2+}. Factors acting through $G\alpha_q$ include cholecystokinin (CCK), acetylcholine and fatty acids. Hormones acting through $G\alpha_s$ e.g. GIP, GLP-1 and adrenalin, cause activation of adenylate cyclase (AC) and increased cAMP levels and subsequent activation of PKA and EPAC2. Activation of $G\alpha_q$ and $G\alpha_s$ stimulates insulin secretion. GPCRs signaling through the $G\alpha_i$ pathway leads to inhibition of AC, reduced cAMP production and inhibition of insulin secretion. Hormones acting through $G\alpha_i$ include ghrelin, SST and NPY. Individual GPCRs can interact with more than one type of Gα subunit. Thus, the effects of any one type of GPCR on insulin secretion may be quite complex [121].

The incretin system and the incretin effect

The incretin hormones GIP and GLP-1 are secreted by enteroendocrine cells and have been intensively studied due to their ability to potentiate

glucose-stimulated insulin secretion. The incretin concept is based on the fact that oral glucose administration is associated with a much greater degree of insulin secretion, compared with that of the isoglycemic intravenous glucose infusion [122, 123]. The incretin effect accounts for 50-70 % of the overall postprandial insulin secretion in healthy subjects [124, 125]. The incretin term was presented by La Barre in 1930 to define the humoral ability of the gut to increase the endocrine secretion from the pancreas [126]. The criteria for incretins was suggested later by Creutzfeldt and required that a gut-derived factor is released in response to nutrients, particularly carbohydrates, and at physiological levels stimulates insulin secretion in the presence of elevated blood glucose levels [127].

GIP and GLP-1 levels increase in response to food intake and stimulate insulin secretion from the beta-cells in a glucose-dependent fashion. Both hormones also have long-term beneficial effects on the preservation or augmentation of pancreatic beta-cell mass, by stimulating beta-cell differentiation from precursors, proliferation of mature beta-cells, and their protection against apoptosis [122]. Interestingly however, genetic disruption of receptors for both GLP-1 and GIP in a mouse model resulted only in a modest effect on glucose homeostasis, No difference in insulin secretion during the intraperitoneal glucose tolerance test [128] or in clamp rates of glucose infusion, endogenous glucose production or disappearance [129] have been reported, suggesting the existence of other peptides with insulinotropic activity.

The GIP secretory defect is not considered to be major in T2D as GIP levels in T2D subjects appears to be unchanged [130] or even slightly increased [131]. Furthermore, obesity has been associated with increased GIP secretion [132-134]. On the other hand, GLP-1 levels were found to be significantly reduced in T2D patients [131, 135, 136]. However, as reviewed by J.J Meier and M.A. Nauck [130], reduced GLP-1 levels were only evident 2-3 hours after a meal and not in the immediate postprandial period. In addition, there is a high individual variation in GLP-1 levels. Thus the mean GLP-1 levels are in fact relatively normal in T2D [130].

Furthermore, the response to GLP-1, including insulinotropic actions, is considered to be largely preserved in T2D patients. This is the basis for the successful use of GLP-1 receptor agonists, as well as inhibitors of the GLP-1 degrading enzyme, dipeptidyl peptidase-4 (DPP4), for the treatment of T2D [130, 134]. On the contrary, the insulinotropic effect of GIP is reduced in T2D subjects [130, 134] and GIP administration to hyperglycemic patients with T2D does not result in a significant glucose-lowering effect [137, 138].

K-cells and GIP

GIP was the first incretin hormone to be identified. It was isolated from extracts of porcine intestine by Brown *et al.* in 1971. Its ability to inhibit gastric acid secretion resulted in its original name: gastric inhibitory polypeptide [139, 140]. Later studies revealed the ability of GIP to stimulate insulin secretion at physiological levels in animals and humans, whereas the inhibitory effect on gastric acid secretion was evident only at pharmacological doses. Therefore GIP was renamed glucose-dependent insulinotropic polypeptide [125]. GIP expression has been described in the intestinal K-cells in both rodents and humans [141, 142]. Recently, GIP expression was demonstrated also in mouse and human pancreatic alpha-cells [46]. In addition, GIP expression has been observed in the submandibular gland [143] and in certain brain regions [144, 145].

The majority of K-cells is located in the duodenum and the proximal jejunum, but a minor part of K-cells is distributed along the entire small intestine. In a subset of intestinal enteroendocrine cells, GIP is colocolized with GLP-1 [142]. Xenin, a 25-amino acid peptide, is also expressed by a subset of K-cells [146].

GIP, like other peptide hormones, undergoes posttranslational processing. GIP is derived from a 153-amino acid phohormone precursor, a proGIP containing signal peptide, an N-terminal peptide, GIP, and a C-terminal peptide [125]. Intestinal proGIP is processed by PC1/3 to yield GIP_{1-42}, whereas in islet alpha-cells PC2 cleavage of proGIP results in GIP_{1-30} that is subsequently converted into $GIP_{1-30NH2}$ [46]. However, 10-15% of K-cells in the small intestine also produce $GIP_{1-30NH2}$, due to the expression of PC2 [147]. Full length GIP_{1-42} is rapidly converted into inactive GIP_{3-42} by DPP4 in a matter of minutes after its release [148].

GIP is released in response to nutrient ingestion, especially fat and glucose. Fat is the most potent stimulator of GIP secretion in humans while carbohydrates are more potent in rodents and pigs [125]. In primary intestinal cell cultures, GIP release has been shown to be triggered by glucose, glutamine and linoleic acid [149]. Basal GIP levels in humans are 0.06-0.1 nmol/L and reach a peak 15-30 min after a meal (0.2-0.5 nmol/L) [125, 150, 151]. The binding of GIP to the GIP receptor (GIPR) on pancreatic beta-cells results in the potentiation of glucose-stimulated insulin secretion (GSIS). It is mediated by an increase in cAMP and the activation of PKA and EPAC2, as well as arachidonic acid release and activation of phospholipase A2. GIP also activates insulin transcription and biosynthesis [152], improves beta-cell survival [153, 154] and stimulates glucagon secretion [155, 156].

L-cells and GLP-1

Observations that immune absorption of GIP from gut extracts failed to completely abolish the incretin effect suggested the presence of additional hormones with incretin properties [122, 157]. The second incretin hormone GLP-1 was discovered after the cloning and characterization of the proglucagon gene. GLP-1 was shown to stimulate GSIS *in vitro* [158] and *in vivo* in humans [159]. GLP-1 is produced by L-cells, mainly located in the distal ileum and colon [125], but also in the upper small intestine [160], in the central nervous system [161] and in the islet alpha-cells [48, 49].

Proglucagon processing by PC1/3 in L-cells in the distal small intestine and colon results in the production of GLP-1, glicentin, oxyntomodulin, and GLP-2 [122].

PC1/3 cleavage leads to formation of $GLP\text{-}1_{17\text{-}37}$ and $GLP\text{-}1_{7\text{-}36NH2}$, the active forms of GLP-1 secreted *in vivo* [162]. Both forms are equally effective in enhancing insulin secretion [163]. The biologically active forms of GLP-1 are converted into inactive $GLP\text{-}1_{9\text{-}37}$ and $GLP\text{-}1_{9\text{-}36NH2}$ by DPP4 in less than 2 min [148, 164]. In addition, GLP-1 is also secreted in the inactive forms of $GLP\text{-}1_{1\text{-}37}$ and $GLP\text{-}1_{1\text{-}36NH2}$ [162].

GLP-1 is secreted in response to carbohydrates, proteins and fats. GLP-1 levels rise rapidly in response to a meal; secretion is biphasic with an early (10-15 min) and later (30-60 min) second phase [165]. Since the majority of L-cells are located in the distal ileum and colon, the early phase of GLP-1 secretion is unlikely to be mediated by direct nutrient sensing, and more likely by the autonomic nervous system. Interestingly, there seems to be an interaction between the two incretins since GIP has been shown to contribute to the first phase GLP-1 secretion [125]. The molecular mechanisms behind the increase in insulin secretion by GLP-1 extensively overlap those of GIP. Binding of GLP-1 to the GLP-1 receptor (GLP-1R) leads to activation of adenylate cyclase and subsequent activation of PKA/EPAC2. Additionally, GLP-1R activation has been shown to increase intracellular Ca^{2+} levels and PKC signaling (reviewed [125]).

GLP-1 has been shown to promote insulin biosynthesis and beta-cell proliferation and to inhibit beta-cell apoptosis. GLP-1 also inhibits glucagon secretion and stimulates SST secretion. Other effects of GLP-1 include inhibition of appetite, gastric acid secretion and gastric emptying. GLP-1 indirectly inhibits hepatic glucose production, and stimulates glucose uptake in adipose tissue and skeletal muscle (reviewed [125]).

CART

Isolation, structure and molecular biology

CART is an acronym for cocaine- and amphetamine-regulated transcript and the name refers to its discovery. *CARTPT* mRNA was discovered by Douglass *et al*. in 1995 as an mRNA being upregulated in rat striatum after acute administration of cocaine or amphetamine [166]. However much earlier, a fragment of the CART peptide was identified in extracts of sheep hypothalamus [167]. The gene encoding the new transcript was given the name *CARTPT* (cocaine-amphetamine regulated transcript prepropeptide (PT)). Following the discovery of *CARTPT* mRNA, the amino-acid sequence of the protein was predicted, including a 27 amino-acid leader sequence, indicating secretion of CART through the regulated secretory pathway. Pairs of basic amino acids in the sequence also suggested the likelihood of cleavage processing [168].

Human CART encoded by the *CARTPT* gene spans approximately 2.0 kb and consists of an approximately 340-nucleotide proximal promoter region, three exons and two introns [169]. In rodents, *Cartpt* mRNA is found in two alternatively spliced forms resulting in production of propeptides of different lengths, named proCART 1-89 and proCART 1-102 (the section encoding amino acids 27-39 is spliced out) [166, 170, 171]. In contrast, only proCART 1-89 is found in humans. Notably, the regions encoding the active parts of the CART peptides lie downstream of the spliced region and are intact in both propeptides. The importance of the different forms of proCART, and if they are differentially regulated, is unknown. However, from the number of cDNA clones identified, one can infer that proCART 1-89 is the more abundantly expressed [166].

Post-translational processing of CART may differ in central and peripheral tissues [172] resulting in several biologically active fragments. The processing of CART is mainly PC-mediated. Using neuroendocrine cell lines expressing different PCs and PC null mice [173], PC2 was shown to play a major role in the generation of CART 55-102/42-89 peptides, while PC1/3 cleavage resulted in the production of the intermediate peptides CART 33-102/10-89. In addition, PC5/6 participates in CART processing sharing specificity with PC2, but not PC1/3 [174]. Moreover, phosphorylation or sulfation of CART was suggested by Thim *et al*. [172].

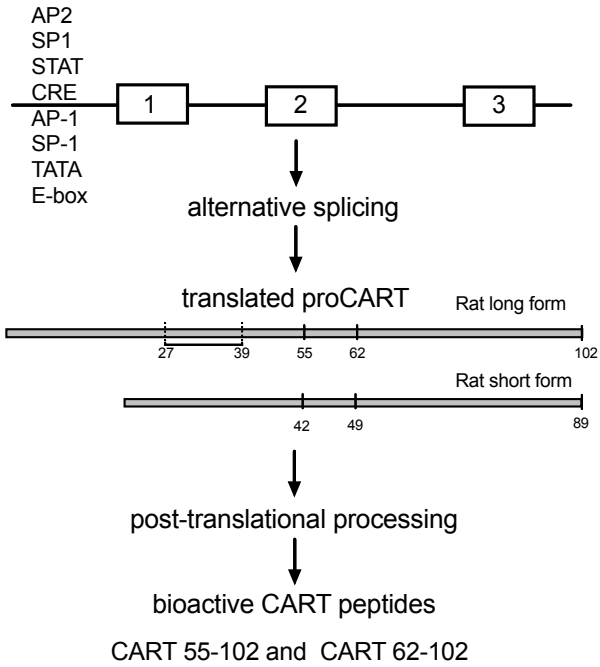


Figure 1. Schematic diagram of the *Cartpt* gene with the predicted transcription factor binding sites. Alternative splicing results in the translation of two proCART poly-peptides, rat long CART and rat short CART that are post-translationally processed into biologically active CART 55-102 and CART 62-102 peptides.

CART peptides are highly conserved across species, sharing 91% nucleotide identity in human and rat coding regions and 98% nucleotide identity in the rat and mouse coding regions. Importantly, this results in 95% amino acid identity between the rat and human proteins, with 100% amino acid identity at the carboxy-termini (exon 3) [169]. This region is found in all CART peptides and contains six cysteine residues forming disulphide bonds that are crucial for the biological activity of CART [175]. The promoter region of the gene is important for *CARTPT* transcriptional regulation. Predicted transcription factor binding sites include TATA box, STAT, cAMP responsive element (CRE), AP1, SP1, AP2, E-box, and Pit-1 sites [176]. *CARTPT* mRNA expression was shown to be regulated by the cAMP/PKA/CREB-dependent signaling pathway since *CARTPT* transcript levels were increased by adenylyl cyclase and PKA activators [176, 177]. Furthermore, CART expression is regulated by CREB in cultured cells [178-180] and CREB binds directly to the CRE-site of the *CARTPT* promoter [181]. In addition, a positive cis-acting element for the

zinc-binding protein factor in the pig *CARTPT* promoter was reported [182]. Regarding negative regulation of *CARTPT* transcription, the promotor neuron-restrictive silencer element (pNRSE) and the first intron NRSE (iNRSE) are involved in the regulation of *CARTPT* transcription. Neuron-restrictive silencer factor (NRSF) binds to NRSEs and recruits HDAC1, mSin3, and CoREST complexes to ensure repression efficiency [183].
In addition to cocaine and amphetamine, *CARTPT* mRNA expression is stimulated by leptin [184], CCK [180], estradiol [185], and glucocorticoids [186].
While much is known about the CART gene and protein structure, processing and regulation, specific CART receptor(s) has not been identified. There is evidence for involvement of pertussis-sensitive Gi/o proteins in mediation of the effects of CART. Pertussis toxin was shown to block CART-stimulated ERK activation in neuronal cell lines [187] and inhibition of voltage-gated Ca^{2+} channels in neurons after CART treatment [188]. However, in beta-cells, CART has been shown to increase cAMP levels in INS-1 (832/13) cells [189] and to potentiate insulin secretion in a cAMP/PKA-dependent manner from INS-1 (832/13) cells and isolated rat islets [190]. This indicates involvement of Gαs rather than Gαi/o in the mediation of the effects of CART in beta-cells and proposes the existence of more than one CART receptor. The identification of the CART receptor is a long-standing research scope of our group. Recently, analysis of RNAseq data of CART-silenced INS-1 (832/13) beta-cells revealed a potential CART receptor candidate. Preliminary data indicates binding of CART to the receptor candidate and involvement of the receptor candidate in CART signaling in beta-cells.

CART Distribution

CART has emerged as a classic brain-gut peptide with a wide range of biological effects as a neurotransmitter and as a hormone [191, 192]. CART expression has been demonstrated in the central, peripheral and enteric nervous systems [168, 184, 192], in adipose tissue [193], as well as in endocrine cells in pancreatic islets [45, 194], the thyroid [195] and the adrenal medulla [195, 196].
In the brain, CART is highly expressed in the arcuate nucleus (Arc), the lateral hypothalamus area (LHA), the paraventricular nucleus (PVN) and the nucleus accumbens (NAc); brain nuclei involved in regulation of food intake [184, 191]. Intracerebro ventricular (ICV) administration of CART peptide inhibits food intake in rats, while administration of neutralizing

antibodies causes the opposite effect [197]. CART expression in the Arc is decreased in food-deprived animals or in animals with disrupted leptin signaling [184]. Moreover, chronic ICV CART peptide administration inhibits food intake, while CART KO mice develop obesity [198, 199]. In humans, a missense mutation within the NH_2-terminal *CARTPT* region is associated with reduced resting energy expenditure and the mutation cosegregates with obesity [200]. Another study has shown that *CARTPT* contributes to the genetic risk for obesity in a Caucasian population [201]. Taken together, there is a body of evidence supporting that CART is an anorexigenic peptide. On the other hand, direct hypothalamic CART administration [202] or overexpression of CART in the PVN [203] leads to hyperphagia indicating difference in the effects of ventricular vs. direct hypothalamic CART administration.

CART is also found in the brain areas associated with reward and reinforcement (NAc, ventral pallidum, amygdala and the ventral tegmental area (VTA)) [204]. In line with this expression pattern, CART was shown to play a role in drug addiction and CART transcript levels were shown to be upregulated in the NAc and downregulated in the VTA in the brain of victims of cocaine overdose [205, 206]. Furthermore, CART KO mice display a decrease in the rewarding properties of psychostimulant drugs and in alcohol consumption [207].

In the GI tract CART expression has been described in the enteric nervous system in rats [192], pigs [195], sheep [208], guinea pigs [209] and humans [210]. Less is known about CART expression in enteroendocrine cells. In sheep, a small population of CART immunoreactive (IR) endocrine cells was detected in the abomasum part of the stomach [208]. In rats, CART-expressing endocrine cells were confined to the antrum and duodenum. The majority of these cells were identical to gastrin-producing G-cells, but a subpopulation in the duodenum remained unidentified [192].

CART is also expressed in both islet endocrine cells and in parasympathetic and sensory nerve fibers innervating the islets. In the developing rat, islet CART is upregulated in all islet cell types, except the ghrelin cells. In developing mice, CART is mainly expressed in the alpha-cells. In the adult normal rat, the intra-islet expression of CART is limited to the somatostatin-producing delta-cells, while in adult mice CART is mainly expressed in nerve fibers as well as in a small subpopulation of beta-cells [45]. In the human pancreas, CART is found in islet alpha- and beta-cells and in cholinergic nerve terminals innervating the islets [211]. Moreover, CART is expressed in islet tumors [212] and neuroendocrine tumors [213], and increased circulating CART levels were detected in

patients with neuroendocrine malignancy [214].

Anti-diabetic effects of CART

For many years, the research focus of our group has been to establish the role of CART in the regulation of glucose homeostasis. Earlier studies in CART KO mice established the importance of CART for maintaining normal islet function. CART KO mice displayed impaired insulin secretion and glucose intolerance due to islet dysfunction [198]. Conversely, mice with beta-cell specific (PDX-1-driven) overexpression of CART (CARTtg mice) have improved glucose-tolerance and increased insulin secretion [211]. Streptozotocin (STZ)-treated CARTtg mice display higher residual insulin secretory capacity and improved glucose elimination (Abels *et al*, manuscript). Furthermore, in a glucose-dependent fashion, exogenous CART was shown to increase insulin secretion from rodent and human islets *in vitro* due to enhanced beta-cell exocytosis. Furthermore, CART inhibits glucagon secretion *in vivo* in mice and *in vitro* in humans and mice, due to reduced alpha-cell exocytosis. Importantly, the insulinotropic effect of CART was retained in T2D donors [211] and diabetic GK-rats [190]. Furthermore, CART protects beta-cells against glucotoxicity-induced cell death *in vitro* in INS-1 (832/13) cells and in cultured rat islets [189]. In line with this, CARTtg mice are partly protected against STZ-induced hyperglycemia (M. Abels *et al*., manuscript). Based on these effects, CART-based agents hold promise for development of antidiabetic therapeutics. In addition, CART further augments GLP-1-enhanced glucose-stimulated insulin secretion *in vitro* and *in vivo* in mice [190, 211], suggesting the possibility of a combinational therapy with CART and GLP-1-based drugs. Furthermore, CART was shown to be upregulated in human T2D islets and in several rodent models of T2D [211, 212], likely as a homeostatic response trying to overcome hyperglycemia. CART expression was increased in rat islets cultured in high glucose and in beta-cells of dexamethasone (DEX)-treated rats. Normalization of blood glucose levels in dexamethasone DEX-treated rats by insulin treatment prevented the upregulation of CART expression [211]. Based on these observations, we believe that increased glucose levels trigger the increased CART expression.

We have also established a role for CART in the insulin target tissues. In adipocytes, CART acts either in concert with or against the action of insulin [193]. Thus, CART decreases basal and isoprenalin-stimulated lipolysis, but potentiates the inhibitory effect of insulin on isoprenaline-induced lipolysis. CART also reduces basal and insulin-induced

lipogenesis. Furthermore, CARTtg mice exhibit increased insulin resistance and altered adipocyte metabolism. The effect of CART overexpression on adipocyte metabolism differed between mice on standard chow and high fat diet (Abels *et al.*, manuscript) indicating the importance of feeding status for the effects of CART and plasticity of the CART system. Another important insulin tissue is skeletal muscle and we have made preliminary observations showing that CART promotes GLUT4 translocation to the plasma membrane with a potency similar to that of insulin (N. Wierup and K. Stenkula, unpublished observations).

In summary, CART is endowed with many effects that are beneficial in a T2D perspective. It is worth to note that many of the described effects of CART resemble those of GLP-1; both hormones increase insulin secretion, protect against beta-cell death and inhibit glucagon secretion. The latter is rather unusual and to date, only GLP-1, SST and insulin have been shown to directly inhibit glucagon secretion [120, 125].
These effects together with the fact that CART can further increase GLP-1-stimulated insulin secretion open up for the possibility of CART-based substances for T2D therapy.

Aims

I. To study the role of endogenous CART in beta-cell function (Paper I).

II. To characterize genetic networks regulated by CART in INS-1 (832/13) cells (Paper II).

III. To establish the role of the CART target gene *Scrt1* in beta-cell function (Paper III).

IV. To examine CART expression in enteroendocrine cells (Paper IV).

V. To establish the role of CART as a regulator of incretin secretion and expression (Paper IV).

Materials and Methods

During my PhD studies, I have worked with *in vitro* and *in vivo models,* and human specimens. In this chapter I describe the main methods I have used in the studies. More detailed information for each method can be found in the original papers.

Human specimens and Immunohistochemistry

Immunohistochemistry is the method of choice for studies of tissue distribution and cellular localization of a target protein. It also enables assessment of morphological and expression changes in the cells, cell populations and tissues after various interventions, which cannot be detected with protein quantification methods such as Western blot or ELISA.

To establish expression of CART in the human upper small intestine and to determine cellular identity of the cells (Paper IV), we performed double- or triple-immunostainings for CART and the endocrine cell marker chromogranin A (CGA), as well as the incretins GIP and GLP-1. Human specimens from duodenum and jejunum were collected during surgery. The study was approved by the Regional Ethical Review Board. Specimens were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.2) before being embedded in paraffin. Sections (6 μm) were cut and mounted on coated slides, deparaffinized and hydrated prior to immunohistochemistry. Normal gross morphology of the specimens was verified using hematoxylin stained sections. The specificity of the CART antibodies was verified with lack of immunoreaction after preabsorption with CART 55-102 peptide (10-100 μg/ml in working dilution), as well as in the GI tract of CART KO mice [213].

Oral glucose tolerance test (OGTT) in mice

In order to investigate potential effects of CART on incretin secretion *in vivo* (Paper IV), I used C57Bl/6J mice. All animal studies were approved by the Animal Ethics Committee in Lund and Malmö, Sweden. Since

CART has previously been shown to have sex-specific effects, experiments were performed in both female and male mice.
Prior to the testing, mice were fasted for 5 hours to minimize individual variation. OGTT was performed in mice anesthetized with Hypnorm/Dormicum and CART was given intravenously 10 min before the OGTT start. Hypnorm is a combination drug consisting of fentanyl (a potent analgesic with a rapid onset and short duration of action) and fluanisone (sedative of the butyrophenone class). Dormicum contains midazolam that acts as a muscle relaxant. CART was given intravenously 10 min before the OGTT start. The chosen anesthesia produces a deep sedation with deep analgesia sufficient to perform short painful procedures. This allowed me to perform OGTT in 20 mice simultaneously with multiple blood sampling from the retroorbital plexus and thus minimizing the variation seen between independent experiments. Previous studied in rodents has shown that the Hypnorm/Dormicum combination causes minor alteration in blood glucose or insulin levels [215]. On the other hand, performing experiments in awake mice may also lead to increased stress resulting in stress hormone release and effects on glucose tolerance. Mice are unlikely to be equally stressed and thereby there is a risk for the introduction of large individual variation in glucose levels. To compensate for the liquid loss, caused by repeated blood sampling, mice were given saline subcutaneously after the test. The mice also were kept warm until the next day to avoid energy consuming hypothermia. Mice were allowed to recover for at least 4 weeks between experiments.

Cell models
During my PhD studies, I have extensively worked with INS-1 (832/13), GLUTag and STC-1 cell lines.

INS-1 (832/13) were derived from the parental INS-1 cells and have been stably transfected with a plasmid containing the human insulin gene. 832/13 was selected as the clone with the highest responsiveness to glucose; insulin secretion was increased 8-fold at 15 mM glucose compared with secretion at 3 mM glucose. The parental INS-1 cells exhibited only a three-fold increase in insulin secretion as glucose was increased from 3 to 15 mM. Importantly, INS-1 (832/13) cells maintain insulin expression and responsiveness to glucose through passages [216]. We used the INS-1 (832/13) cell line to study the effect of CART KD and SCRT1 KD on insulin expression and secretion (Paper I and III respectively).

GLUTag cells (kindly provided by Prof. D.J. Drucker, Mount Sinai Hospital, Toronto, Canada), originally isolated from a glucagon-producing enteroendocrine tumor in mice [217], were used for studies of GLP-1 expression and secretion. In addition to GLP-1, GLUTag cells express GLP-2, glicentin/oxyntomodulin, CCK, neurotensin, glucagon and SST [218]. The GLP-1 secretion response to various stimuli in GLUTag cells is similar to that seen in non-immortalized murine L-cells [219]. We used GLUTag cells to study regulation of CART gene expression as well as the effect of CART KD and exogenous CART treatment on GLP-1 expression and secretion (Paper IV).

The polyhormonal secretin tumor cell line STC-1 [220] (a kind gift from Prof. J.Y. Scoazec, Edouard Herriot Hospital, Lyon, France) was used as a K-cell model for studies of GIP expression. STC-1 cells express multiple peptide hormones, including GLP-1, GLP-2, glicentin/oxyntomodulin, CCK, gastrin, CGA, neutotensin, VIP, glucagon, SST and amylin [218, 221, 222]. Notably, only about 7% of the STC-1 cells contain GIP immunoreactivity [221] and therefore GIP levels in the media during secretion experiments are too low to be measured with the commercially available assays. STC-1 cells were used to study regulation of CART gene expression as well as the effect of CART KD and exogenous CART treatment on GIP mRNA expression (Paper IV).

siRNA-mediated gene silencing

RNA interference (RNAi), employing double-stranded short interfering RNAs (siRNAs), is a technique used for the silencing of genes. After entering the cell, the short double-stranded RNA sequence binds to the protein machinery of the RNA induced silencing complex (RISC) and activates the catalytic component of RISC. This leads to the cleavage of complementary mRNA transcripts and results in mRNA degradation and the subsequent prevention of protein translation. However, it is now a well-known fact that in addition to the on-target silencing, siRNAs may also produce off-target effects [223]. To distinguish target-specific effects from off-target effects, we routinely used several siRNAs in parallel experiments for each gene to be targeted. Another approach is to pool multiple siRNAs for selective reduction of off-target effects or to perform rescue experiments [223].

To target siRNA into the cells, we employed lipid-mediated transfection. We used lipofectamine RNAiMAX that is a cationic lipid formulation, *i.e.*

liposomes that form complex with anionic RNA. The liposomes/RNA complex fuse with the plasma membrane delivering siRNA into the cytoplasm via endocytosis [224]. In my studies, I used siRNA to silence CART expression in INS-1 (832/13) (Paper I and II), GLUTag and STC-1 cells (Paper IV) and SCRT1 expression in INS-1 (832/13) cells (Paper III).

Hormone secretion assays
To assess the effect of CART KD (Paper I) and SCRT1 KD (Paper III) on beta-cell function, I performed insulin secretion studies. INS-1 (832/13) cells were washed twice and incubated for two hours in 2.8 mM glucose followed by 1 h- or 15-min stimulation with various stimulants.

In GLUTag cells I performed GLP-1 secretion studies (Paper IV). Cells were washed twice and incubated with secretagogues for two hours.

Secretion studies include multiple steps of solution changes. In my opinion, it is key to perform secretion studies only in cells that look perfect, evenly distributed in the well and to be very gentle during replacing media/solutions to avoid cell detachment. I believe, that treating the cells like your "babies" results in reproducible solid data.

Assessment of gene and protein expression
To assess messenger RNA (mRNA) levels after gene silencing or treatment with exogenous stimuli, I used quantitative real-time polymerase chain reaction (qPCR) using TaqMan probes. TaqMan is designed to increase qPCR specificity. Taq polymerase cleaves a labeled probe hybridized to the complementary target sequence. After cleavage, the probe loses its quencher leading to the fluorescence signal that is quantified and represents product accumulation (Life Technologies).

To semi-quantify the effect of gene silencing on target protein expression, I performed Western blot. I first separated proteins by gel electrophoresis based on size and then transferred the proteins from the gel to the nitrocellulose membrane by means of electrophoresis. During the next step, the membrane was incubated with antibodies with specificity for the target protein. Bound proteins were visualized using secondary antibodies and detection reagents. Importantly, CART is a peptide with molecular weight of only around 5 kDa. Therefore, I used 10-20% Tris-Tricine gels, designed for the separation of small proteins and peptides, during the gel electrophoresis step.

I also used ELISAs for quantification of protein content in blood samples, cell lysates and secretion assay supernatants. ELISA is the first-choice method for studies of protein levels in the blood, culture media or in secretion assay buffers after incubation with cells. The ELISAs that I have used are based on the sandwich technique, in which monoclonal antibodies against the target protein are bound to the wells of a microwell plate. Proteins in the sample react with these antibodies and are quantified in the next step using secondary antibodies and a detection reagent, e.g. peroxidase-conjugated anti-insulin antibodies for insulin ELISA.

Cell viability, cytotoxicity and apoptosis measurement

The ApoTox-Glo Triplex assay (Promega, Madison, WI) was used to assess the effects of CART silencing on INS-1 (832/13) cell survival (Paper I). The assay allows for simultaneous measurement of viability, cytotoxicity and apoptosis in the same sample. Viability and cytotoxicity are measured by fluorescent signals produced when either live-cell or dead-cell proteases cleave added substrates (the substrates have different excitation and emission spectra). Apoptosis was measured by the addition of caspase-3/7 substrate, which is cleaved in apoptotic cells to produce a luminescent signal.

Results and Discussion

Aim I. The role of endogenous CART in beta-cell function

Previous studies from the lab have established that administration of exogenous CART increases insulin secretion and protects against glucotoxicity-induced beta-cell death [189, 190, 194, 198, 211]. Mice with global disruption of CART display impaired insulin secretion *in vivo* and in isolated islets [198]. On the other hand, islet CART expression is increased in T2D patients and diabetic rodent models [190, 211]. However, the function of endogenous beta-cell CART has remained largely unknown. Therefore, my first aim was to target CART specifically in the beta-cell to assess potential impact on insulin secretion and beta-cell viability.

Effect of CART silencing on insulin secretion and exocytosis

To establish the role of endogenous CART in beta-cells, we silenced the expression of CART in INS-1 (832/13) insulin-producing cells using siRNA (**Figure 2 A** and **B**). First, I examined insulin secretion 1 h after stimulation with a battery of key insulin secretagogues. In line with the reduced glucose-stimulated insulin secretion observed in CART KO mouse islets [198], CART silencing reduced insulin secretion stimulated by glucose alone (**Figure 2 C**). In addition, CART silencing reduced insulin secretion stimulated with cAMP-elevating agents, K^+, and the mitochondrial fuel α-KIC (**Figure 2 C**). This may suggest that CART acts at the late events of insulin secretion, e.g. exocytosis. Therefore, we next assessed the effect of CART silencing on first phase insulin secretion during 15-min static incubations. The stimulatory effect of K^+ at 2.8 mM glucose during the 15-min incubation was reduced in CART-silenced cells (**Figure 2 D**). As ATP is an important trigger and amplifying factor of insulin secretion [225, 226], we hypothesized that reduced levels of ATP may mediate the effects of CART silencing on insulin secretion. Therefore, I measured ATP levels after 15-min incubation with glucose,

with or without inhibitors of mitochondrial ATP synthesis. Indeed, ATP levels in CART-silenced cells were decreased at both 2.8 mM and 16.7 mM glucose (**Figure 2 E**). Interestingly, there was no effect of CART KD on ATP content after uncoupling with FCCP and blocking ATP synthesis with OM, suggesting that CART KD leads to a reduction in ATP synthesis. These data gain support from observations showing that CART preserves ATP levels after ischemic conditions in neurons [227].

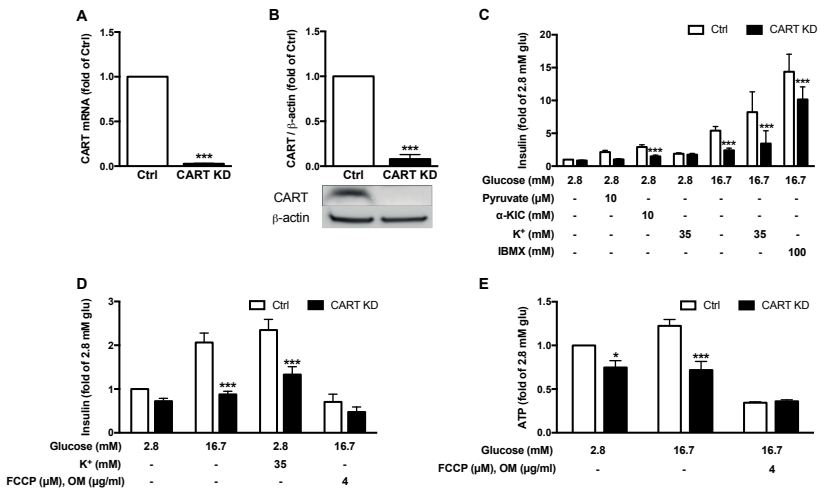


Figure 2. CART KD reduces insulin secretion and ATP levels in beta-cells
Decreased *Cart* mRNA (**A**) and CART protein (**B**) expression after CART KD in INS-1 (832/13) cells relative to control (scrambled siRNA, Ctrl) (n=4 and n=6 respectively). CART KD reduces insulin secretion stimulated by 16.7 mM glucose, alpha-ketoisocaproate (alpha-KIC), K^+ (at 16.7, but not 2.8 mM glucose) and 3-isobutyl-1-methylxanthine (IBMX) during 1h static incubations (**C**). During 15 min static incubations CART KD reduces insulin secretion stimulated by 16.7 mM glucose, as well as K^+-stimulated insulin secretion at 2.8 mM glucose (**D**). The latter effect was abolished by addition of the uncoupler carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) and the ATP synthase inhibitor oligomycin (OM) (n=3-9). CART KD reduces ATP content at 2.8 and 16.7 mM glucose. The effect was abolished by addition of FCCP and OM (**E**). (n=3). Data presented as mean±SEM. * $p<0.05$, *** $p<0.001$.

As insulin secretion was found to be reduced after CART KD in INS-1 (832/13) cells and as exogenous CART increases beta-cell exocytosis in mouse islets [211], we next assessed the effect of CART KD on exocytosis. We used the standard whole-cell configuration of the patch-clamp technique. Exocytosis, measured as changes in membrane capacitance, was elicited by a train of ten 500-ms depolarizing pulses from −70 to 0 mV that evokes the influx of Ca^{2+} needed to stimulate granule

fusion. CART KD had no effect on the total increase in capacitance (**Figure 3 A-C**), or on Na^+ or Ca^{2+} currents evoked by the train of depolarizations (**Figure 3 D and E**), but CART KD caused a robust reduction in the capacitance increase evoked by the two first depolarizations (**Figure 3 A-C**). This is believed to represent exocytosis of primed granules in the readily releasable pool (RRP) of granules [228, 229]. It should be noted that the patch clamp experiments were performed in the presence of ATP, suggesting the involvement of additional mechanisms to ATP behind the reduced insulin secretion after CART KD. In order to understand the basis for the reduced exocytosis after CART KD, we next measured expression of genes important for exocytosis.

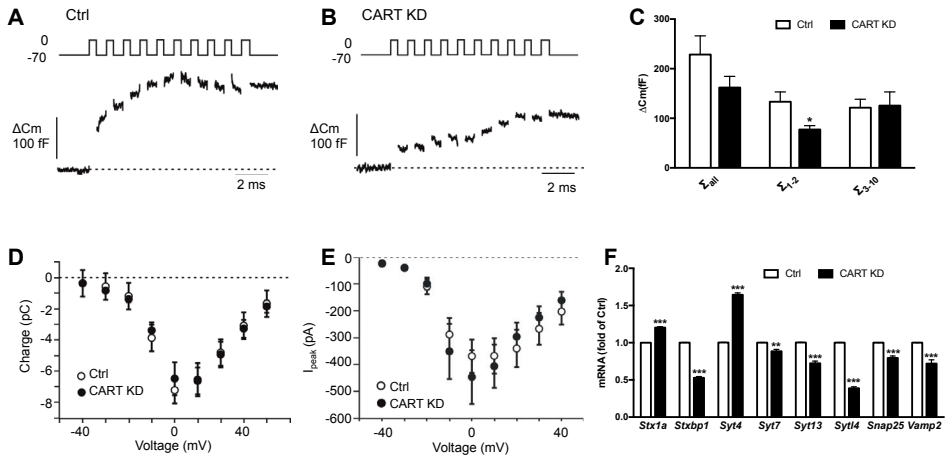


Figure 3. CART KD reduces beta-cell exocytosis and alters expression of exocytotic genes

Representative traces of depolarization-induced exocytosis, measured as changes in cell membrane capacitance (ΔCm), in Ctrl (scrambled siRNA) (**A**) and CART KD (**B**) cells. Quantification of the capacitance changes induced by all depolarizations ($\sum_{all}$), by the first two depolarizations ($\sum_{1\text{-}2}$), corresponding to the readily releasable pool of granules, as well as by depolarizations 3-10 ($\sum_{3\text{-}10}$) (**C**). Sustained charge (Q)–voltage (V) (**D**) and peak current (I_{peak})–voltage (V) (**E**) relationship measured in single Ctrl (scrambled siRNA) or CART KD cells. Effect of CART KD on expression of exocytotic genes (**F**). Data are expressed as mean±SEM of 9–12 cells in each group (A-E) or 3 biological experiments (F). * $p<0.05$, *** $p<0.001$.

CART KD reduced expression of *Stxbp1*, *Syt7*, *Syt13*, *Sytl4*, *Snap25* and *Vamp2,* while *Stx1a* and *Syt4* expression was increased (**Figure 3 F**), suggesting an important role of CART in regulating the expression of proteins involved in granular fusion [102].

SNAP25 has previously been demonstrated to have an important role in beta-cell exocytosis through mechanisms that are independent of Ca^{2+} influx [107, 108]. STX1a and STXBP1 are critical for the formation of granular docking sites [106] and STXBP1 together with SYTL4 are important for docking [110]. Thus, the CART KD-induced reduction of *Stxbp1* expression and increase in *Sytx1a* and *Sytl4* expression is more complex, but might explain the lack of effect on granular docking and mobilization (represented by an increase in membrane capacitance by the later depolarizations of the train). Furthermore, expression of the synaptotagmin genes *Syt7* and *Syt13* was slightly reduced after CART KD. Deletion of SYT7 in knock-out animals leads to reduced first phase insulin release [230], and first phase insulin secretion has been suggested to be associated with the release of RRP granules [226]. Not much is known about the function of SYT4 and SYT13 in beta-cell exocytosis, but silencing of *Syt13* in INS-1 (832/13) cells reduces glucose stimulated insulin secretion and the expression of *SYT4*, *SYT7* and *SYT13* is reduced in islets of T2D donors [231].

Effect of CART silencing on expression of insulin and genes involved in insulin secretion and production

The reduced insulin secretion observed after CART KD could potentially also be related to impaired insulin transcription and processing. Therefore, we examined insulin gene and protein expression after CART KD in INS-1 (832/13) cells. This revealed that *Ins1* and *Ins2* mRNA levels and proinsulin protein levels were decreased after CART KD (**Figure 4 B)**. To assess whether the decreased insulin content is responsible for the reduction in insulin secretion seen after CART KD, we repeated the 1-h insulin secretion experiments and normalized insulin secretion to insulin content of the cells. This revealed that CART KD reduced insulin secretion also when adjusted for insulin content (**Figure 4 E**). Thus, in line with the effect of CART KD on exocytosis, the reduction in insulin content does not alone explain the reduced GSIS. To gain further insight into the role of CART in other processes important for proper insulin release, we also examined how CART KD affects expression of genes encoding GLUT2, PCs and glucokinase. We found that CART KD caused reduced expression of *Glut2* and *Gck* (**Figure 4 F**). This suggests a role for CART in glucose sensing. The present finding of reduced expression of *Glut2* is supported by previous observations in islets from CART KO mice [198].

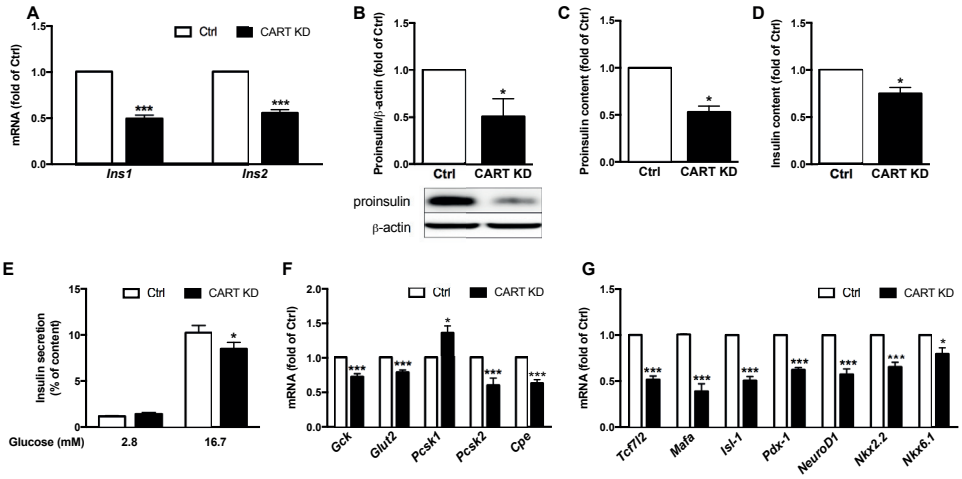


Figure 4. CART KD decreases expression of insulin and important beta-cell genes
Reduced *Ins1* and *Ins2* gene expression after CART KD (n=6) compared to Ctrl (scrambled siRNA) (**A**). Band densitometry of Western blot and representative blot showing reduced proinsulin protein levels after CART KD (n=7) (**B**). Reduced proinsulin (**C**) and insulin (**D)** content after CART KD, measured using ELISA (n=6). Insulin secretion (% of content) at 2.8 mM and 16.7 mM glucose in Ctrl (scrambled siRNA) and CART KD (n=6) as indicated (**E**). CART KD affects the expression of genes involved in glucose transport and sensing, as well as insulin processing (n=4-6) (**F**). Decreased *Tcf7l2*, *Mafa*, *Isl1*, *Pdx*-1, *NeuroD1, Nkx2.2 and Nkx6.1* expression after CART KD (n=3-6) (**G**). Data presented as mean±SEM. *, $p<0.05$; ***, $p<0.001$.

We also studied the expression of the prohormone convertases 1/3 and 2 (*Pcsk1* and *Pcsk2*) and carboxypeptidase E (*Cpe*) that cleave proinsulin to insulin and C-peptide [232]. We found that CART KD decreased the expression of *Pcsk2* and *Cpe*, but increased the expression of *Pcsk1* (**Figure 4 F**). The fact that CART KD had a divergent effect on the expression of these enzymes speaks against a major role for CART as a regulator of proinsulin processing. I rather believe that the observed reduced insulin synthesis and secretion is a consequence of reduced expression of key beta-cell transcription factors, in turn affecting insulin transcription. Thus, CART KD reduced expression of *Mafa*, *Pdx-1*, *Isl1*, *NeuroD1*, *Nkx2.2* and *Nkx6.1* (**Figure 4 G**) that can activate insulin transcription [85, 96, 233-237]. The effect on *Pdx-1* expression is supported by previously shown reduced PDX-1 protein expression in the islets of CART KO mice [198]. CART KD also reduced *Tcf7l2,* the number one risk gene for T2D thus far identified. TCF7L2 has recently been shown to bind to *Isl1* and to control expression of *Pdx-1*, *NeuroD1*,

Nkx6.1 and *Mafa* transcription factors, and key beta-cell genes (including *Pcsk1* and *Pcsk2*) regulating insulin processing, maturation and secretion [85]. Thus, there is a large overlap in the genes affected by CART and TCF7L2 KD in INS-1 (832/13) cells suggesting that TCF7L2 and CART activate similar pathways. Although the exact mechanisms are difficult to dissect due to the lack of identified CART receptors [191, 238], our data position CART as an important regulator of beta-cell transcription.

CART has previously been shown to protect beta-cells from glucotoxicity-induced cell death [27]. To rule out the observed effects of CART KD being secondary to reduced cell viability, we next assessed whether silencing of CART would influence beta-cell survival using the ApoTox-Glo Triplex assay. This assay allows for the simultaneous measurement of cell viability, cytotoxicity and apoptosis. CART KD caused an increase in caspase 3/7 activity (**Figure 5 B**), without affecting viability (**Figure 5 A**) or cytotoxicity (**Figure 5 C**). Thus, our data suggest cytoprotective properties of CART in beta-cells. However, the observed increase in apoptosis was not reflected in beta-cell viability and likely does not explain the effects of CART KD on decreased insulin secretion and expression.

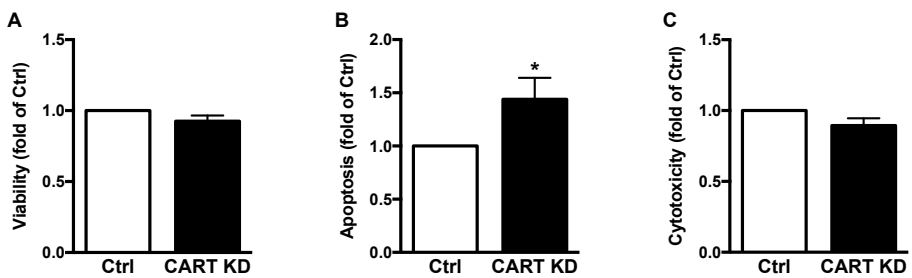
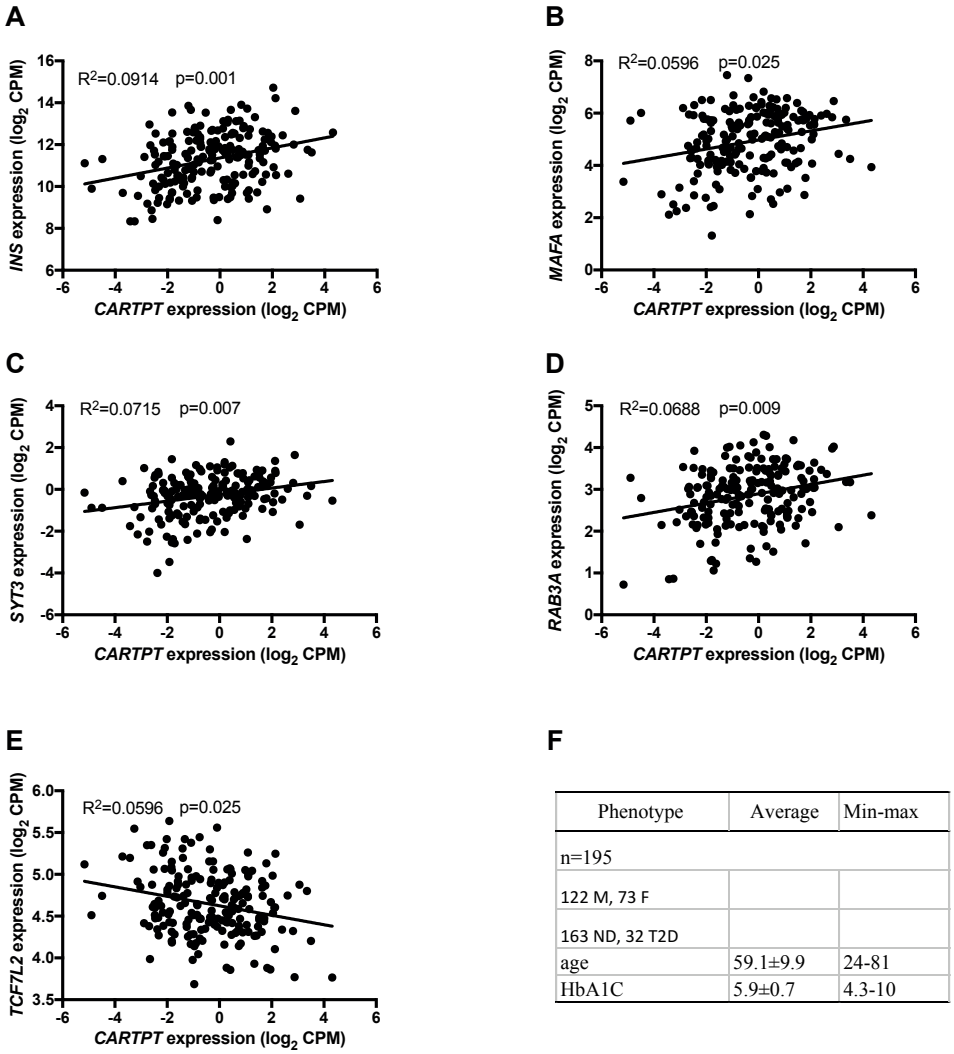


Figure 5. CART KD increases beta-cell apoptosis but does not alter cell viability or cytotoxicity
CART KD in INS-1 (832/13) cells results in increased apoptosis (**B**), without affecting cell viability (**A**) or cytotoxicity (**C**) (n=6) compared to Ctrl (scrambled siRNA). Data presented as mean±SEM. * $p<0.05$.



F

Phenotype	Average	Min-max
n=195		
122 M, 73 F		
163 ND, 32 T2D		
age	59.1±9.9	24-81
HbA1C	5.9±0.7	4.3-10

Figure 6. *CARTPT* mRNA correlates with important beta-cell genes in human islets
CARTPT expression correlates positively with *INS* (**A**)*, MAFA* (**B**)*, SYT3* (**C**) and *RAB3A* (**D**), but negatively with *TCF7L2* (**E**) in RNAseq data from 195 human islet donors. Islet donor characteristics (**F**). T2D: type 2 diabetic donors; ND: non-diabetic donors; M: male; F: female. P-value was adjusted for multiple testing.

Finally, we used RNA-sequencing data from 195 islet donors to assess whether our findings in INS-1 (832/13) cells are relevant for the human situation. Indeed, highly significant correlations of *CARTPT* with *INS, MAFA, TCF7L2, SYT3* and *RAB3A* were evident (**Figure 6**). A role for

CART in human beta-cell function is further supported by previous data showing that CART increases insulin secretion in human islets and that islet CART expression is increased in T2D patients [211].

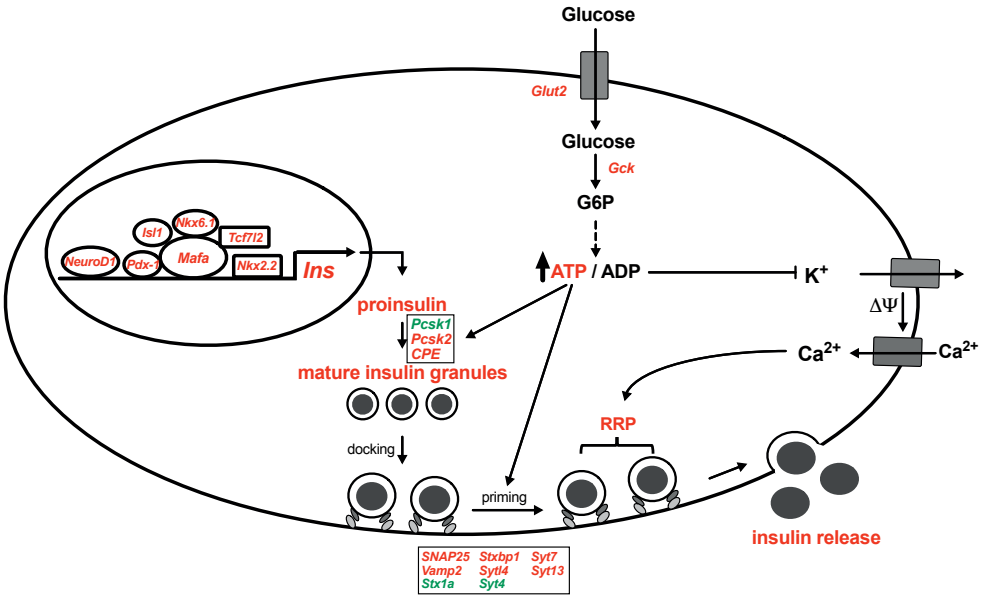


Figure 7. Cartoon illustrating a model of how endogenous beta-cell CART regulates insulin secretion and production. Red indicates reduced, and green - increased expression or levels after CART KD.

In summary, CART in beta-cells acts as a regulator of insulin secretion at multiple levels (**Figure 7**). CART silencing in INS-1 (832/13) cells resulted in decreased insulin secretion and insulin expression, reduced intracellular ATP levels and exocytosis. Therefore, I conclude that endogenous beta-cell CART plays important roles in the regulation of beta-cell function and suggest that CART expression is necessary for maintaining the beta-cell phenotype.

Aim II. Characterization of genetic networks regulated by CART in INS-1 (832/13) cells

I have established that silencing of endogenous CART in INS-1 (832/13) cells results in impaired insulin secretion and expression paralleled with decreased expression of *Tcf7l2*, *Mafa*, *Isl1*, *Pdx-1, NeuroD1, Nkx2.2* and

Nkx6.1. We therefore assumed the involvement of additional genes important for the beta-cell phenotype in the observed effects in INS-1 (832/13) cells. To understand the mechanistic basis for the effect of CART on beta-cell function, we characterized the effects of CART silencing on gene expression in INS-1 (832/13) cells using RNA-sequencing.

RNAseq analysis revealed that of all 19696 genes that had enough read coverage to be analyzed, 2495 genes were upregulated and 2531 genes were downregulated after CART silencing (adjusted p-value of ≤0.05 for multiple comparisons). The fact that CART silencing affected the expression of more than 25% of the detected genes suggests that CART plays an important role in beta-cells. In comparison, silencing of the transcription factor TCF7L2 in INS-1 (832/13) cells resulted in differential expression of 33% of genes [85].
The top 50 downregulated genes and top 50 upregulated genes are presented in **Table 1 A** and **B** respectively. *AABR07068253.1* (similar to Signal Recognition Particle Alu Rna Binding Heterodimer, Srp914) was identified as the most strongly upregulated gene after CART KD (**Table 1 B**). The heterodimeric protein complex SRP9/SRP14 (SRP9/14) is a component of the signal recognition particle (SRP) [239, 240] and is involved in the regulation of translation where it is required for elongation of polypeptide chains or possibly for inhibition of polysome formation and initiation of protein synthesis [241]. The Signal Recognition Particle 14, *Srp14* appeared as the third gene most strongly upregulated by CART KD (**Table 1 B**). *Jun* was the second most upregulated gene after CART KD (**Table 1 B**). It encodes the part of AP-1 transcription factor complex that has been implicated in cell differentiation, proliferation and oncogenic transformation [242]. The top ten most upregulated genes include also *Sfta2* (Surfactant-Associated Protein 2), *F3* (Coagulation Factor III), *LOC100910404* (actin), *Ndnf* (neuron derived neurotrophic factor), *Ldhb* (Lactate Dehydrogenase B) and *Rhob* (Ras Homolog Family Member B).
Cartpt appeared as the most strongly downregulated gene after CART KD, thus confirming KD efficiency (**Table 1 A**). The top downregulated genes include gene-candidate tumor suppressor *Adam 11* [243], *Pak3* (P21 (RAC1) Activated Kinase 3), a serine/threonine kinase of the PAK family that is important for synaptic plasticity in mice. Importantly, PAK3 has recently been shown to act downstream of Ngn3 and to regulate beta-cell differentiation and function [244] (**Table 1 A**). The top ten downregulated genes also include *Ppp1r17* (Protein Phosphatase 1 Regulatory Subunit 17/G-substrate), *Btbd17* (BTB Domain Containing 17), *Trim72* (Tripartite

Motif Containing 72), *Igsf11* (Immunoglobulin Superfamily Member 11), *Lmbrd2* (LMBR1 Domain Containing 2), *Hpca* (Hippocalcin), *AC142182.1* and *Scrt1* (Scratch Family Transcriptional Repressor 1).

Our main aim was to gain insight into the mechanisms behind the effect of CART on insulin secretion [190, 211, 245]. Therefore, we next assessed genes with established importance for insulin secretion and exocytosis using a gene list acquired from Kyoto Encyclopedia of Genes and Genomes (KEGG) [246]. The top three differentially expressed genes related to insulin secretion and exocytosis (based on adjusted p-value) were *Stxa1*, *Gnas* and *Stxbp1* (**Table 2**). Syntaxin 1 (STX1) is mainly known to be a positive regulator of beta-cell exocytosis [107], but has previously also been shown to act as a negative regulator of insulin release [109]. STX1 and STXBP1 are critical for the formation of granular docking sites [106]. STXBP1 has also been reported to be important for docking [110]. We have previously shown that CART KD in INS-1 (832/13) cells leads to decreased exocytosis of insulin granules in the RRP and affects expression of *Stx1a* and *Stxbp1* [245]. Thus, CART appears to be essential for proper exocytosis in INS-1 (832/13) cells. *Gnas* is an imrinted gene with important roles in fetal growth and postnatal energy metabolism. *Gnas* generates multiple gene products including transcripts encoding the G protein α-subunit Gsα that couples many receptors for hormones, neurotransmitters and other extracellular signals to adenylyl cyclase [247]. *Gnas* was downregulated after CART KD in INS-1 832/13 cells and mice with beta-cell-specific Gsα deficiency display reduced beta-cell mass and develop insulin-deficient diabetes [248].

Table 1. The top 50genes displaying differential expression after CART KD in INS-1 (832/13) cells sorted based on fold-change (FC): **A**. Downregulated. **B**. Upregulated.

A

gene	FC	p	padj
Cartpt	**5,36**	7,10E-31	8,09E-29
Adam11	4,80	3,16E-31	3,69E-29
Pak3	4,58	1,70E-52	6,44E-50
Ppp1r17	4,43	1,20E-24	8,28E-23
Btbd17	3,91	1,66E-30	1,77E-28
Trim72	3,80	1,45E-17	5,58E-16
Igsf11	3,74	2,60E-31	3,07E-29
Lmbrd2	3,57	1,53E-78	1,68E-75
Hpca	3,56	3,10E-23	1,93E-21
AC142182.1	3,53	1,03E-44	2,49E-42
Scrt1	3,44	1,44E-38	2,47E-36
Ifi27l2b	3,42	1,14E-25	8,36E-24
Gpd1l	3,21	1,36E-75	1,34E-72
Lgi2	3,13	1,09E-42	2,19E-40
Panx2	3,11	2,14E-50	7,32E-48
Adamts8	3,10	3,06E-17	1,13E-15
Pdzd3	3,09	4,51E-16	1,49E-14
Mustn1	3,07	3,06E-17	1,13E-15
Neurod4	3,06	1,96E-21	1,03E-19
Gpr63	3,04	1,50E-16	5,23E-15
Akap2	3,00	8,27E-15	2,44E-13
Rarres2	2,98	9,64E-14	2,49E-12
AABR07038971.1	2,96	2,13E-12	4,65E-11
Nrep	2,93	1,88E-54	8,06E-52
Ifng	2,91	1,94E-28	1,76E-26
Gm2a	2,90	1,69E-53	6,79E-51
Glipr2	2,90	2,81E-26	2,17E-24
Lynx1	2,89	1,14E-30	1,26E-28
Grm4	2,87	3,58E-18	1,43E-16
Trpv1	2,86	6,12E-18	2,43E-16
Rpl3l	2,85	3,05E-74	2,86E-71
Enpp2	2,82	3,78E-46	9,81E-44
Msn	2,80	9,29E-27	7,44E-25
Il22	2,80	3,89E-17	1,43E-15
Baiap3	2,79	1,13E-93	3,18E-90
Tmem178a	2,74	8,31E-14	2,17E-12
St3gal1	2,73	3,09E-65	1,79E-62
Ctnna2	2,73	3,35E-12	7,19E-11
Cx3cl1	2,72	9,70E-28	8,34E-26
Gng8	2,72	8,32E-13	1,89E-11
RX	2,71	2,80E-93	6,90E-90
Mbnl2	2,71	2,21E-71	1,82E-68
Blnk	2,69	1,93E-09	2,82E-08
Cyp4f1	2,67	1,12E-12	2,50E-11
Spock2	2,67	1,29E-52	4,96E-50
Bend6	2,66	2,71E-54	1,14E-51
Gng7	2,65	1,80E-36	2,73E-34
Cops8	2,64	3,46E-98	1,36E-94
Cd40	2,62	8,09E-13	1,84E-11
Nfic	2,60	1,03E-85	1,69E-82

B

gene	FC	p	padj
AABR07068253.1	0,22	8,26E-82	1,02E-78
Jun	0,23	1,51E-43	3,32E-41
Srp14	0,24	4,76E-58	2,34E-55
Sfta2	0,29	3,53E-14	9,67E-13
Apmap	0,31	1,51E-53	6,20E-51
F3	0,33	1,19E-48	3,67E-46
LOC100910404	0,34	3,03E-27	2,51E-25
Ndnf	0,34	2,24E-13	5,49E-12
Ldhb	0,34	4,25E-14	1,15E-12
Rhob	0,35	1,32E-49	4,40E-47
Ffar4	0,36	2,27E-14	6,31E-13
Akap12	0,37	5,29E-70	4,01E-67
P2ry10	0,37	6,47E-10	1,02E-08
Ube2m	0,38	4,12E-158	8,11E-154
Ripply3	0,39	1,61E-44	3,83E-42
AABR07060293.1	0,40	3,57E-53	1,40E-50
Acpp	0,40	1,20E-15	3,83E-14
Il10ra	0,40	7,06E-25	4,98E-23
Tmprss13	0,40	5,25E-24	3,40E-22
Rsad2	0,40	5,03E-14	1,34E-12
Zdhhc9	0,40	2,97E-66	1,83E-63
Insig1	0,40	5,48E-58	2,63E-55
Gpm6a	0,41	1,97E-14	5,53E-13
Potec	0,41	2,66E-08	3,29E-07
Psma1	0,41	3,20E-97	1,05E-93
Rn50_X_0744.6	0,41	2,29E-10	3,85E-09
Dpy19l2	0,42	1,90E-09	2,79E-08
Gstm2	0,42	3,60E-44	8,15E-42
Tle3	0,43	7,34E-31	8,31E-29
Tmem38a	0,43	2,61E-24	1,76E-22
Vom2r12	0,44	3,25E-84	4,92E-81
Tex264	0,44	1,09E-44	2,62E-42
Hsbp1	0,45	2,19E-21	1,14E-19
L1cam	0,45	6,20E-19	2,65E-17
Gad2	0,45	2,58E-15	7,98E-14
Sec23a	0,45	3,58E-36	5,30E-34
Sgpp2	0,45	1,10E-21	5,96E-20
Plagl2	0,45	6,10E-77	6,32E-74
Celf5	0,46	4,75E-28	4,23E-26
Tnfrsf19	0,46	9,99E-07	9,43E-06
Ntm	0,46	1,86E-12	4,09E-11
Nrp2	0,46	5,32E-13	1,24E-11
Map9	0,46	1,54E-45	3,90E-43
Tsx	0,46	3,90E-10	6,35E-09
Fam89a	0,47	1,06E-29	1,06E-27
Styk1	0,47	3,15E-06	2,71E-05
AABR07058699.2	0,47	2,53E-24	1,73E-22
Hcar1	0,47	3,15E-06	2,71E-05
S100a5	0,48	1,09E-06	1,02E-05
AC128848.1	0,48	5,66E-12	1,18E-10

Genes related to insulin secretion significantly affected by CART KD also include exocytotic genes e.g. synaptotagmins, *Vamp2*, *Snap25*, *Rab3a*; beta-cell transcription factors e.g. *Tcf7l2*, *Pdx-1*, *Isl1*, *Neurod1*, *Nkx2*.2 and genes important for insulin processing and secretion e.g. *Pcsk1*, *Pcsk2*, Cpe, *Abcc8* and *Kcnj11* (**Table 2**). Many of these genes were previously shown to be regulated by CART KD and are likely to mediate the effect of CART KD on insulin granule exocytosis and insulin expression [245].

Abcc8 and *Kcnj11* encoding SUR1 and KIR6.2, forming ATP-sensitive potassium (K_{ATP}) channels in beta-cells, were among the top regulated insulin secretion genes (**Table 2**). K_{ATP} channel closure is an essential step in stimulus secretion coupling and sulfonylurea drugs, such as tolbutamide and glibenclamide, closing K_{ATP} channels are potent insulin secretagogues and widely used T2D drugs [225]. Expression of both *Abcc8* and *Kcnj11* was increased after CART KD suggesting that CART KD may result in increased K_{ATP} channel activity and decreased insulin secretion, contributing to the reduced insulin secretion observed after CART KD in INS-1 (832/13) cells. Intriguingly, CART is overexpressed in beta-cells of patients with nesidioblastosis (N. Wierup and J. Rahier, unpublished observations), where in many cases *Abcc8* and *Kcnj11* gene mutations have been identified to be causal [249].

In order to further explore cellular functions potentially affected by CART silencing, we performed pathway analysis of the differentially expressed genes after CART KD. Differentially expressed genes (adjusted *p*-value of ≤ 0.05) and their corresponding fold-change were uploaded into the Ingenuity Pathway Analysis (IPA®) software (Qiagen, Redwood City, CA) for analysis. This revealed that CART KD affected 281 canonical pathways ($p<0.05$; not shown). Molecular mechanisms of cancer, PKA signaling and cardiac hypertrophy signaling were most significantly affected. Insulin receptor signaling, AMPK signaling and T2D diabetes signaling were among the 10% most regulated pathways. We focused on the T2D signaling to gain insight into potential mechanisms underlying impaired insulin secretion after CART KD in INS-1 (832/13) cells.

Table 2. The top 50 differentially expressed insulin secretion/exocytosis genes after CART KD in INS-1 (832/13) cells sorted based on adjusted p-value (padj). FC=fold-change.

gene	FC	p	padj
Stx1a	0,70	2,46E-26	1,92E-24
Gnas	2,49	3,28E-21	1,68E-19
Stxbp1	1,57	7,50E-20	3,47E-18
Pcsk1	0,55	1,51E-18	6,27E-17
Syt4	0,68	2,90E-18	1,18E-16
Neurod1	1,27	5,87E-12	1,21E-10
Camk2a	2,22	9,27E-12	1,88E-10
Camk2b	0,73	2,91E-11	5,52E-10
Syt10	2,38	6,42E-11	1,16E-09
Cacna1d	1,19	7,24E-11	1,29E-09
Nkx6-1	0,68	1,59E-08	2,05E-07
Syt14	1,36	4,47E-08	5,37E-07
Rab3a	0,80	5,72E-08	6,72E-07
Syt7	0,76	8,55E-08	9,72E-07
Nkx2-2	1,34	1,24E-07	1,38E-06
Gip	0,85	1,36E-07	1,50E-06
Adcyap1r1	1,58	5,21E-07	5,16E-06
Kcnj11	0,68	5,69E-07	5,59E-06
Abcc8	0,78	3,01E-06	2,61E-05
Camk2d	1,18	3,60E-06	3,07E-05
Kcnmb2	1,41	7,41E-06	5,93E-05
Plcb1	0,83	9,57E-06	7,50E-05
Syt15	1,46	1,14E-05	8,82E-05
Ryr2	1,36	1,14E-05	8,82E-05
Adcy5	1,34	1,28E-05	9,76E-05
Adcy6	1,23	1,74E-05	1,29E-04
Adcy3	1,28	3,33E-05	2,33E-04
Atf6b	0,89	5,07E-05	3,43E-04
Atp1a4	1,25	6,49E-05	4,27E-04
Atf2	0,84	1,04E-04	6,53E-04
Pclo	1,32	1,76E-04	1,04E-03
Pdx1	1,14	1,81E-04	1,06E-03
Creb5	0,56	4,38E-04	2,34E-03
Prkacb	0,85	5,46E-04	2,83E-03
Isl1	1,23	7,49E-04	3,75E-03
Prkca	0,82	9,53E-04	4,61E-03
Sytl4	1,30	9,83E-04	4,74E-03
Cacna1s	0,84	1,09E-03	5,19E-03
Kcnn1	0,79	1,15E-03	5,43E-03
Rims2	1,14	1,50E-03	6,82E-03
Rims2	1,14	1,50E-03	6,82E-03
Atp1a3	1,24	1,82E-03	8,07E-03
Cpe	1,17	1,85E-03	8,18E-03
Pcsk2	1,25	4,55E-03	1,75E-02
Plcb3	1,14	4,93E-03	1,88E-02
Syt12	1,29	6,28E-03	2,31E-02
Syt12	1,29	6,28E-03	2,31E-02
Mafa	1,50	6,96E-03	2,53E-02
Tcf7l2	1,32	7,42E-03	2,67E-02
Creb3l1	1,31	1,31E-02	4,33E-02
Adcy9	1,18	1,36E-02	4,48E-02
Vamp2	1,11	1,51E-02	4,88E-02

Table 3. Genes previously shown to be regulated by CART KD in INS-1 (832/13) cells.

	FC	pvalue	padj
Slc2a2	0,92	3,62E-01	5,56E-01
Gck	1,00	9,45E-01	9,74E-01
Pcsk1	0,55	0,00E+00	6,27E-17
Pcsk2	1,25	4,55E-03	1,75E-02
Cpe	1,17	1,85E-03	8,18E-03
Ins1	1,31	8,19E-02	1,89E-01
Ins2	1,07	6,37E-01	7,89E-01
Tcf7l2	1,32	7,42E-03	2,67E-02
Mafa	1,50	6,96E-03	2,53E-02
Nkx6-1	0,68	0,00E+00	2,05E-07
Nkx2-2	1,34	0,00E+00	1,38E-06
Isl1	1,23	7,50E-04	3,75E-03
Neurod1	1,27	0,00E+00	1,21E-10
Pdx1	1,14	1,80E-04	1,06E-03
Stx1a	0,70	0,00E+00	1,92E-24
Syt4	0,68	0,00E+00	1,18E-16
Syt7	0,76	0,00E+00	9,72E-07
Syt13	1,14	7,32E-02	1,73E-01
Stxbp1	1,57	0,00E+00	3,47E-18
Vamp2	1,11	1,51E-02	4,88E-02
Snap25	1,01	9,41E-01	9,72E-01

The prediction tool of the IPA software suggested a glucotoxicity-mediated increase in ROS that via down-regulation of beta-cell transcription factors would lead to impaired insulin secretion and apoptosis in beta-cells (**Figure 8**). Notably, exogenous CART has previously been shown to protect beta-cells from glucotoxicity-induced cell death [189] and CART KD resulted in increased apoptosis (Paper I). In neuronal cells, CART has been shown to act as an antioxidant, protecting mitochondrial DNA, cellular proteins and lipids from the oxidative action of hydrogen peroxide [250]. Thus, ROS may play a role in CART signaling in beta-cells.

In addition, the glucose-induced repressor of insulin gene transcription *Cebpb* [251] was suggested to be a downstream target of CART. Furthermore, KIR6.2 and SUR1, encoded by *Abcc8* and *Kcnj11* (appeared also among the top regulated insulin genes), were upregulated after CART KD and predicted to be involved in CART action (**Figure 8**). *Cebpb* expression was decreased after CART KD. CEBPB is a transcriptional repressor with regulatory functions in beta-cells. CEBPB expression is increased in clonal beta-cells cultured at high glucose and in islets in T2D

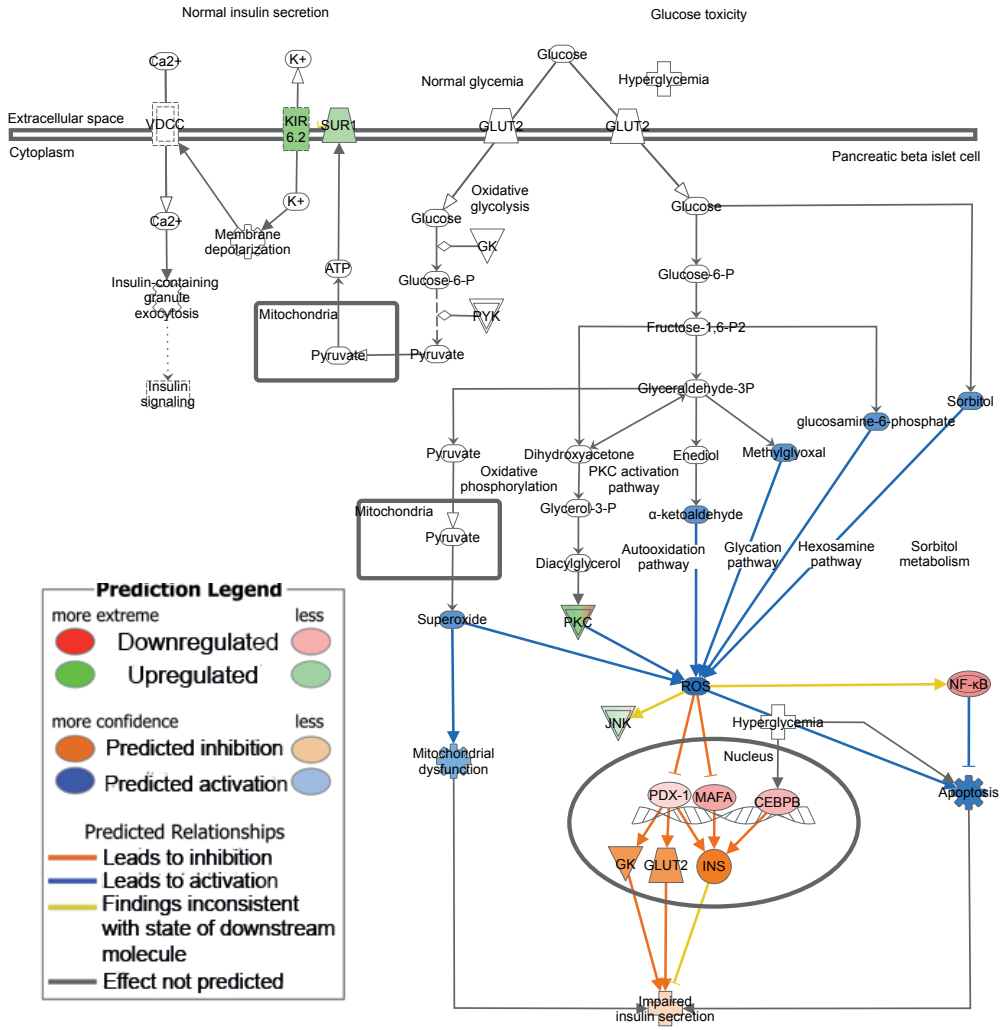


Figure 8. Elements of the canonical T2D signaling pathway in pancreatic beta-cells that are affected by CART KD in INS-1 (832/13) cells as predicted by pathway analysis. Elements of the pathway displayed in red are downregulated and in green are upregulated; intensity of the color corresponds to the magnitude of the effect on gene expression. Predicted inhibition of the pathway elements is shown in orange and predicted activation is shown in blue; intensity of the color corresponds to the confidence of the predicted effect. Orange arrows indicate predicted downstream inhibition; blue arrows indicate predicted downstream activation.

models [243, 252, 253]. It is also involved in the repression of insulin transcription under supraphysiological glucose concentrations [251]. CEBPB has also been shown to induce cell cycle arrest and senescence of tumor cells [254] and to promote mammary epithelial cell differentiation [255]. However, opposite to what we expected, *Cebpb* expression was increased after CART silencing in INS-1 (832/13) cells, perhaps as a compensatory mechanism to restore impaired insulin transcription.

Thus, the predicted action of CART in the present study is in agreement with our previously reported functional data [245] (Paper I) and studies in CART KO mice [198] showing that downregulation or deletion of CART leads to impairment in GSIS and insulin expression. We also confirmed altered expression of most of the previously qPCR identified CART regulated genes (**Table 3**) [245] confirming the reliability of the RNAseq data.
It should be mentioned that the current analysis of the differentially expressed genes was performed based on p-value without introducing the fold-change cut-off. This approach was selected in order to characterize all the CART KD-activated changes and gene networks. Future studies using the fold-change cut-off will most likely be useful for identifying the most prominent effects and pathways.
In summary, we have characterized CART-activated genes and pathways in INS-1 (832/13) cells. CART was shown to modulate expression of genes with important roles in insulin secretion, expression and granule exocytosis. Molecular mechanisms of cancer, PKA signaling, cardiac hypertrophy, insulin receptor signaling, AMPK signaling and T2D diabetes signaling were among the most regulated pathways affected by CART KD. We have also identified novel possible mediators of the CART-effects in beta-cells. Functional follow-up studies are needed to understand whether the predicted pathways translate into functional pathways.

Aim III. The role of the CART target gene *Scrt1* in beta-cell function

The RNAseq analysis of CART-silenced INS-1 (832/13) cells identified Scratch Family Transcriptional Repressor 1 (*Scrt1*) as a highly CART-regulated transcript (Paper II). *Scrt1* was the most strongly downregulated

transcription factor transcript and was on 10th place among the most downregulated genes. The expression of *Scrt1* was decreased by 70% after CART silencing. The literature on SCRT1 is very limited and the function of SCRT1 has only been described in neuronal cells. SCRT1 is described as a transcriptional repressor belonging to the SNAIL family of zinc finger transcription factors. This family of transcription factors binds to E-box motifs in gene promotors and has been shown to be major regulators of epithelial-to-mesenchymal transition [256]. Recently, SCRT1 was shown to regulate the onset of neuronal migration via the downregulation of E-cadherin [257]. Hitherto, SCRT1 expression has not been described in islets and there is no data available on the role of SCRT1 in beta-cell function. The observation that *Scrt1* is strongly regulated by CART, may suggest that SCRT1 is a mediator of the effects of CART. Therefore, we decided to assess a potential role of SCRT1 in beta-cell function.

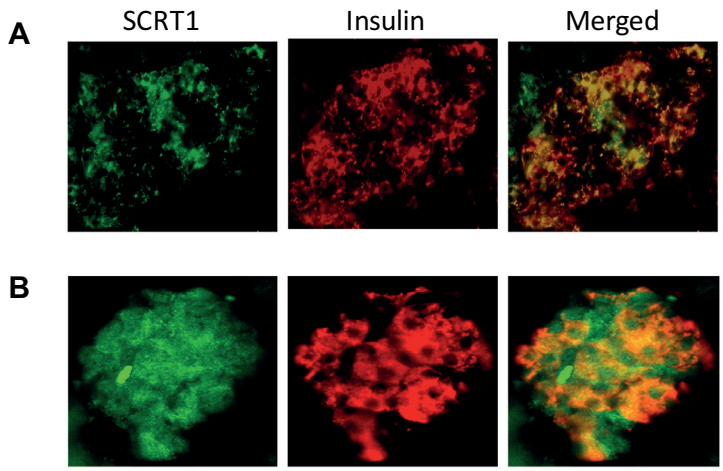


Figure 9. Human and mouse pancreatic sections, double immunostained for SCRT1 (green) and insulin (red). SCRT1 is expressed in human (**A**) and mouse (**B**) beta-cells.

First, we aimed to study the expression pattern of SCRT1 in human and rodent islets. By the use of immunohistochemistry, we showed that SCRT1 expression was evident in beta-cells of humans, rats and mice (**Figure 9** and **12**). Using available RNAseq data on transcription levels in sorted human alpha-, beta- and exocrine cells from a previously published study by Bramswig *et al.* [258], we found that *SCRT1* expression was 10-fold higher in beta-cells compared with exocrine cells.

Next, we established the function of SCRT1 in beta-cells using siRNA silencing of SCRT1 expression (SCRT1 KD) in INS-1 (832/13) cells. As the insulin gene promoter contains E-boxes and SCRT1 is a transcription factor that binds to E-boxes, we assumed that SCRT1 KD would affect insulin transcription. Indeed, SCRT1 KD resulted in reduced mRNA expression of *Ins1* and *Ins2*, as well as the human *INS* expressed in the INS-1 (832/13) cells [216] (**Figure 10 A**). Reduced insulin transcription was paralleled by a 15% reduction in insulin content (**Figure 10 B**). These effects are reminiscent of those of CART silencing in the same cell line (Paper I). Next, we examined expression of the transcription factors that were downregulated after CART KD and were suggested to be key to the observed reduced beta-cell function. In line with the effect of CART KD, SCRT1 KD in INS-1 (832/13) cells reduced expression of *Tcf7l2*, *Isl1*, *Pdx-1*, *Neurod1* and *Mafa* (**Figure 10 C**).

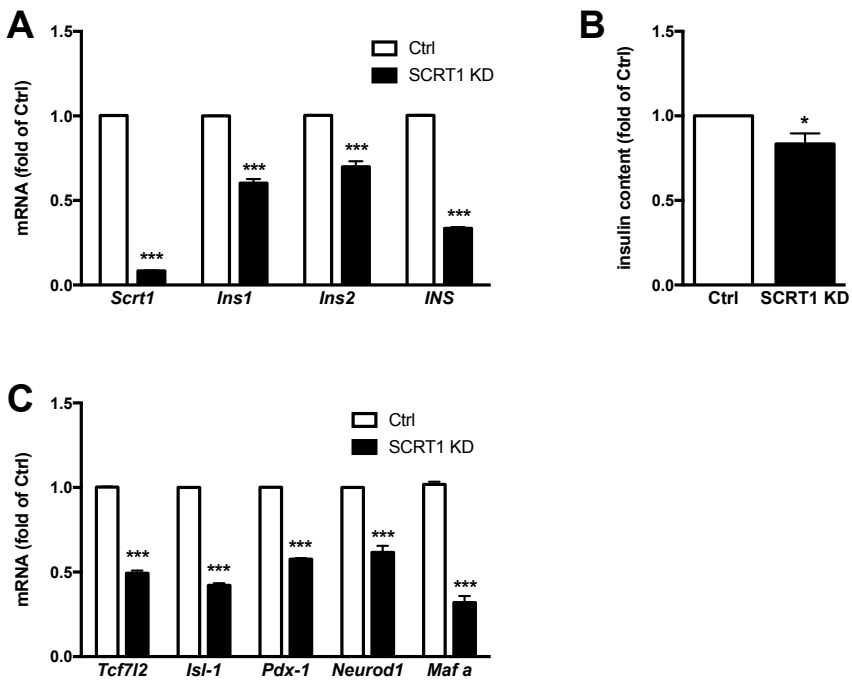


Figure 10. SCRT1 KD reduces insulin and transcription factor expression in INS-1 (832/13) cells

Decreased *Ins1, Ins2* and *INS* gene expression after SCRT1 KD compared with scrambled siRNA (Ctrl) (**A**). Reduced insulin content after SCRT1 KD (**B**). SCRT1 KD in INS-1 (832/13) cells reduces the expression of beta-cell transcription factors (**C**). Data presented as mean±SEM. *, $p<0.05$; ***, $p<0.001$.

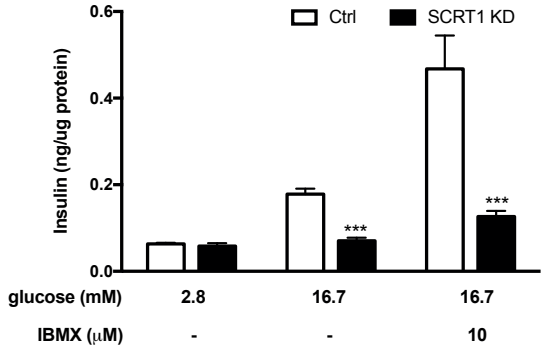


Figure 11. Insulin secretion is reduced in INS-1 (832/13) cells after SCRT1 KD
SCRT1 KD reduces insulin secretion stimulated by 16.7 mM glucose and by 16.7 mM glucose combined with 3-isobutyl-1-methylxanthine (IBMX) during 1h static incubations. Data presented as mean±SEM. ***, $p<0.001$.

The observed effect on these transcription factors prompted studies of insulin secretion after SCRT1 KD in INS-1 (832/13) cells. During 1-h static incubations, SCRT1 KD had no effect on basal insulin secretion (2.8 mM glucose), but resulted in robustly reduced insulin secretion in cells stimulated with 16.7 mM glucose or 16.7 mM glucose combined with IBMX (**Figure 11**). Although we have not normalized insulin secretion to insulin content of the cells, our data suggest that the decreased insulin secretion observed after SCRT1 KD is due to a combination of impaired release and reduced insulin production. The ability of SCRT1 to interact with E-box sequences of DNA promoters may suggest direct binding of SCRT1 to the insulin promotor and regulation of insulin transcription. SCRT1 may also interact with histone deacetylases and modify chromatin structure, as was shown for SNAIL-regulated E-cadherin expression [259]. Additionally, SCRT1 may regulate insulin transcription and secretion by binding to the E-box-containing promotors of beta-cell transcription factors e.g. PDX-1 [260]. ChIP experiments are key for identifying transcriptional targets of SCRT1.

To investigate a potential role of SCRT1 in T2D, we assessed SCRT1 expression in diabetic DEX-treated rats [190, 211]. Interestingly, SCRT1 was found to be translocated from the nucleus to the cytosol in DEX rats (**Figure 12**). This may suggest that SCRT1 expression is regulated by glucose. To test this, we assessed *SCRT1* expression after culture at different concentrations of glucose. This revealed that SCRT1 mRNA expression was reduced after culture at high glucose in INS-1 (832/13) cells (**Figure 13 A**), as well as in human islets (**Figure 13 B**). The

observed translocation of SCRT1 suggests impairment in the shuttling of SCRT1 from the cytosol to the nucleus in the diabetic state. This speaks in favor of decreased SCRT1 transcriptional activity in T2D beta-cells. Interestingly, SCRT1 in INS-1 (832/13) cells is translocated to the cytoplasm after culture at low glucose and low serum concentrations (S. Chriett, unpublished observations). This was somewhat unexpected in view of our data obtained in diabetic DEX rats, and may suggest that other factors than glucose, e.g. cellular stress may inhibit the nuclear translocation of SCRT1. Furthermore, phosphatase inhibitor treatment also results in inhibition of SCRT1 translocation to the nucleus (S. Chriett, unpublished observations) suggesting that SCRT1 phosphorylation is required for the nuclear shuttling and perhaps also for the transcriptional activity of SCRT1.

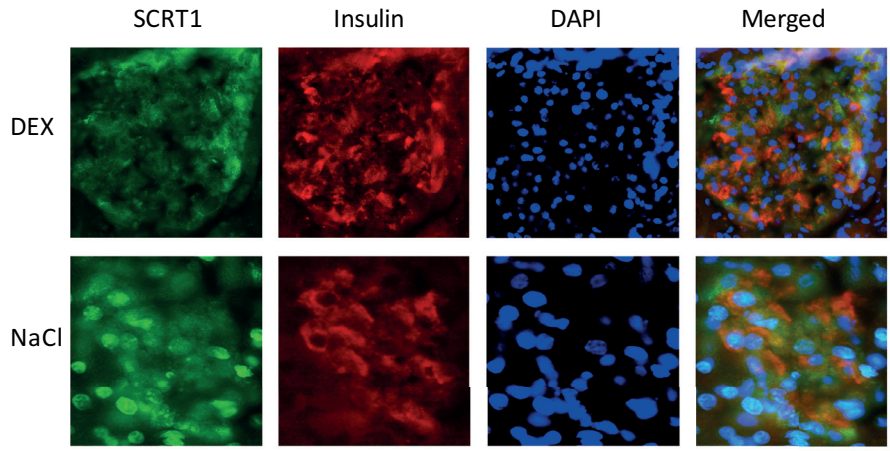


Figure 12. Double immunostaining for SCRT1 (green) and insulin (red), DAPI (blue) in rat pancreatic sections. SCRT1 is translocated to the cytoplasm in the diabetic DEX-treated rats (upper panel) compared with saline-treated control rats (lower panel).

Based on our findings suggesting that SCRT1 is a positive regulator of several important beta-cell genes, the cytoplasmic accumulation of SCRT1 is compatible with impaired beta-cell function. The inhibitory effect of high glucose on *SCRT1* mRNA levels in INS-1 (832/13) cells and human islets suggests that reduced SCRT1 expression may be involved in mediating the detrimental effects of glucotoxicity (and oxidative stress) on insulin expression and secretion as has been shown for MAFA and PDX-1 [261, 262]. Interestingly, knockdown of Snail resulted in increased cell death of breast cancer cells under glucose starvation, while Snail overexpression potentiated cell survival. The effects were shown to be

mediated by changes in ROS and NAPDH levels and Snail was suggested to confer cell survival against oxidative stress. Mechanistically, Snail suppresses aerobic glycolysis and switches glucose flux towards the pentose phosphate pathway (PPP), therefore providing a survival advantage to breast cancer cells [263]. It remains to be tested whether SCRT1 has similar actions on glucose metabolism in beta-cells. Notably, PPP that was previously thought to be inactive in beta-cells, has recently been linked to the regulation of insulin secretion [264].

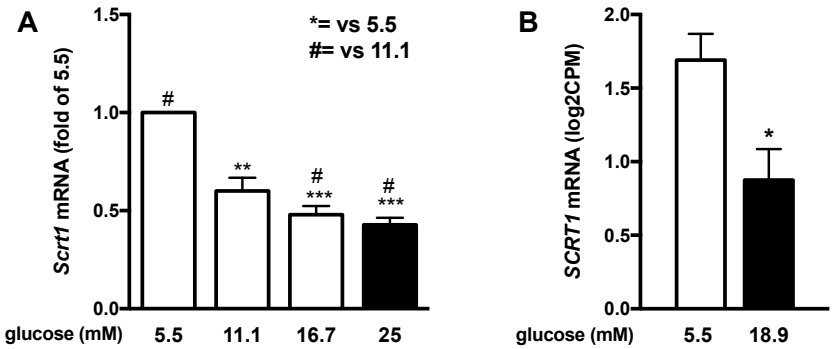
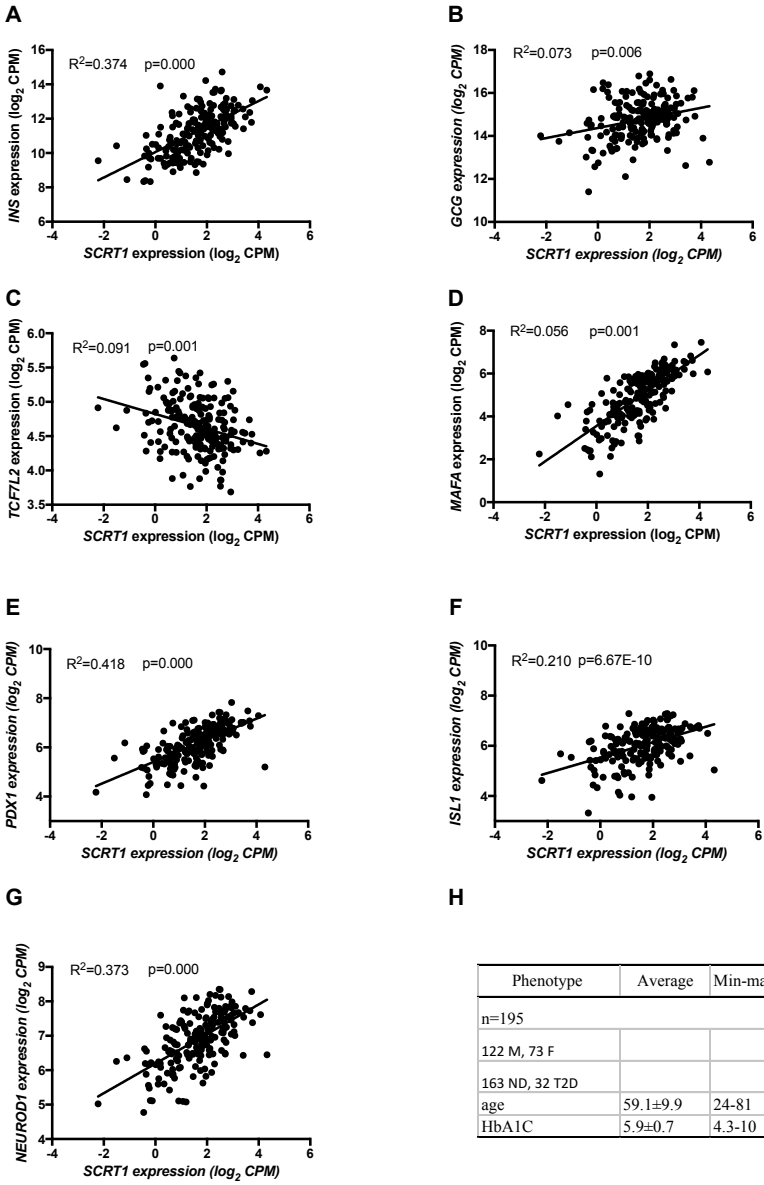


Figure 13. *SCRT1* expression is regulated by glucose in INS-1 (832/13) cells and in human islets

Scrt1 mRNA in INS-1 (832/13 cells) is reduced after 24-h culture in 11.1, 16.7 and 25 mM glucose compared with cells cultured at 5.5 mM glucose (**A**). *SCRT1* mRNA levels are decreased after culture in 18.9 mM glucose in human islets (**B**). Data presented as mean±SEM. *, $p<0.05$; #, $p<0.05$.

Next, we wanted to study if SCRT1 plays a role in human islet function. To this end, we performed correlation analysis to assess whether *SCRT1* expression correlates with genes important for beta-cell function in available RNAseq data from 195 human islets donors [57]. *SCRT1* expression correlated positively with *INS* (**Figure 14 A**), *GCG* (**Figure 4 B**), and the transcription factors *MAFA*, *PDX1*, *ISL1* and *NEUROD1* (**Figure 14 D-G**), but negatively with *TCF7L2* (**Figure 14 C**). In addition, *SCRT1* expression correlated with the expression of genes involved in regulation of insulin expression and processing, as well as glucose sensing (**Table 4**). *SCRT1* expression also correlated with the expression of genes important for beta-cell exocytosis, including syntaxins and synaptotagmins (**Table 4**). Thus, although we have no causal evidence, these observations suggest that SCRT1 may be of importance also in human beta-cells.



Phenotype	Average	Min-max
n=195		
122 M, 73 F		
163 ND, 32 T2D		
age	59.1±9.9	24-81
HbA1C	5.9±0.7	4.3-10

Figure 14. *SCRT1* expression correlates with important beta-cell genes in human islets

SCRT1 expression correlated positively with *INS* (**A**), *GCG* (**B**), and the transcription factors *MAFA*, *PDX1*, *ISL1* and *NEUROD1* (**D-G**), but negatively with *TCF7L2* (**C**) in RNAseq data from 195 human islet donors. Islet donor characteristics (**F**). T2D: type 2 diabetic donors; ND: non-diabetic donors; M: male; F: female. P-value was adjusted for multiple testing.

Table 4. *SCRT1* expression correlates with important beta-cell genes in human islets in RNAseq data from 195 human islet donors.

gene	Correlation coefficient, r	nominal p-value	adjusted p-value	Significant	
				Nominal	Bonferroni corrected
INS	0,61171637610504800000	0,00E+00	0,00E+00	YES	YES
GCG	0,27042207135525500000	1,32E-04	5,66E-03	YES	YES
TCF7L2	-0,30290594997542300000	1,68E-05	7,21E-04	YES	YES
MAFA	0,75223245923285800000	0,00E+00	0,00E+00	YES	YES
MAFB	0,63987836869840400000	0,00E+00	0,00E+00	YES	YES
PDX1	0,64651155780333800000	0,00E+00	0,00E+00	YES	YES
NKX6-1	0,63807549688688400000	0,00E+00	0,00E+00	YES	YES
NKX2-2	0,65907956523921000000	0,00E+00	0,00E+00	YES	YES
ISL1	0,45865351369767100000	1,55E-11	6,67E-10	YES	YES
NEUROD1	0,61102945811802600000	0,00E+00	0,00E+00	YES	YES
GCK	0,59629857488426700000	0,00E+00	0,00E+00	YES	YES
PCSK1	0,30975371093719300000	1,05E-05	4,52E-04	YES	YES
PCSK2	0,52684337934012700000	2,55E-15	1,10E-13	YES	YES
SLC2A1	0,13341505643962900000	6,30E-02	2,71E+00		
CPE	0,28258666300342400000	6,26E-05	2,69E-03	YES	YES
RAB3A	0,74312810464892300000	0,00E+00	0,00E+00	YES	YES
RIMS1	-0,22753176806150300000	1,38E-03	5,93E-02	YES	
RIMS2	0,37416539014208000000	7,11E-08	3,06E-06	YES	YES
SNAP25	0,31647605798905200000	6,56E-06	2,82E-04	YES	YES
STX1A	0,57368138788642900000	0,00E+00	0,00E+00	YES	YES
STXBP1	0,66107676506956800000	0,00E+00	0,00E+00	YES	YES
STXBP2	0,06219694763002030000	3,88E-01	1,67E+01		
STXBP3	-0,32144135499038900000	4,61E-06	1,98E-04	YES	YES
SYT1	0,32861600911042600000	2,73E-06	1,17E-04	YES	YES
SYT10	0,05229318022450990000	4,68E-01	2,01E+01		
SYT11	0,35047958259272100000	5,09E-07	2,19E-05	YES	YES
SYT12	0,02794119324123840000	6,98E-01	3,00E+01		
SYT13	0,45690278262524000000	1,89E-11	8,15E-10	YES	YES
SYT14	0,38898427499130300000	1,91E-08	8,22E-07	YES	YES
SYT15	-0,11502613961408700000	1,09E-01	4,70E+00		
SYT16	0,25104468413515200000	4,00E-04	1,72E-02	YES	YES
SYT17	0,61988400577715300000	0,00E+00	0,00E+00	YES	YES
SYT2	0,03640647887749210000	6,13E-01	2,64E+01		
SYT3	0,47054067599410500000	3,89E-12	1,67E-10	YES	YES
SYT4	0,29563763514274600000	2,72E-05	1,17E-03	YES	YES
SYT5	0,57246343635445800000	0,00E+00	0,00E+00	YES	YES
SYT6	-0,28806437486998200000	4,43E-05	1,91E-03	YES	YES
SYT7	0,68545205742487800000	0,00E+00	0,00E+00	YES	YES
SYT8	0,04959393576460860000	4,91E-01	2,11E+01		
SYT9	0,25067843967808700000	4,08E-04	1,76E-02	YES	YES
SYTL4	-0,15813648431835200000	2,72E-02	1,17E+00	YES	
UNC13B	-0,02650857445099300000	7,13E-01	3,07E+01		
VAMP2	0,30124957656741200000	1,87E-05	8,05E-04	YES	YES

Importantly, for both *SCRT1* and *CARTPT,* there is an overlap in the correlation with key beta-cell genes in human islets (*INS*, *MAFA*, *SYT3*, *TCF7L2*, *RAB3A*), with a greater number of genes correlated with *SCRT1*. This may suggest that the action of SCRT1 is downstream of CART and that SCRT1 is a more direct regulator of these genes.

Additionally, silencing of either SCRT1 or CART in INS-1 (832/13) cells impaired insulin expression and secretion and reduced the expression of

the same beta-cell transcription factors. Thus, it is tempting to speculate that SCRT1 is a mediator of the effects of CART on insulin in INS-1 (832/13) cells. On the other hand, when comparing the effect of CART KD with that of SCRT1 KD some differences were apparent. Silencing of CART resulted in a robust decrease in insulin transcription and insulin content, with a modest effect on insulin secretion (Paper I), whereas SCRT1 KD had a robust effect on insulin secretion, and transcription, but only a modest effect on insulin content was seen. This indicates differences in the mode of action and that SCRT1 is likely not the only mediator of the CART effects.

In summary, we have shown that SCRT1 expression is evident in beta-cells of humans, rats and mice. SCRT1 was found to be translocated from the nucleus to the cytosol in diabetic rats and *SCRT1* mRNA expression to be reduced by increasing glucose concentrations in INS-1 (832/13) cells and human islets. Furthermore, SCRT1 KD resulted in reduced insulin secretion, as well as insulin gene and protein expression in parallel with reduced expression of key beta-cell transcription factors. The mechanisms by which SCRT1 exerts its effects in the beta-cell are not known. However, SNAIL family members bind to E-box motifs in gene promotors and are shown to be major regulators of epithelial-to-mesenchymal transition. Cell communication and synchronization are important for beta-cell function and E-cadherin is necessary for proper insulin response [265, 266]. Therefore, SCRT1 may regulate beta-cell function via direct binding to the insulin promotor or via its target genes e.g. E-cadherin. Our preliminary data indicates reduced *Cdh1* expression after SCRT1 KD and our ChIP data suggest binding of SCRT1 to the E-boxes in the *Ins1* promoter (S. Chriett, unpublished observations) and thus supporting a direct regulation of insulin transcription by SCRT1.
We conclude that SCRT1 is a novel CART-regulated beta-cell transcriptional regulator with potentially important roles as a regulator of beta-cell function.

Aim IV. CART expression in enteroendocrine cells

In the GI tract, CART is widely expressed in the enteric nervous system, but less is known about CART expression in enteroendocrine cells, particularly in the normal human GI-tract. Based on observations on unidentified CART-expressing cells [192] in the GI tract, we decided to

decipher the cellular identity of these cells in the human duodenum and jejunum.

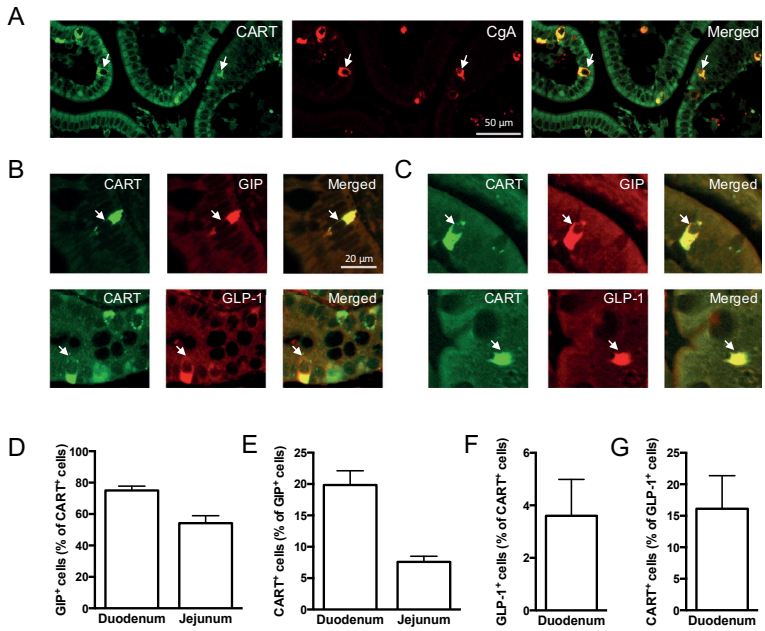


Figure 15. CART is expressed in subpopulations of human K-cells and L-cells Colocalization of CART and the endocrine cell marker chromogranin A (CgA) in human duodenal mucosa (**A**). Colocalization of CART and GIP or GLP-1 in duodenal (**B**) and jejunal mucosa (**C**). Arrows indicate colocalization. Scale bar in B for B and C. Quantification of double positive cell number relative to total number of $CART^+$ (**D**) or GIP^+cells (**E**) in duodenum and jejunum. Quantification of double positive cell number relative to total number of $CART^+$ (**F**) or $GLP\text{-}1^+$ cells (**G**) in duodenum.

CART immunoreactive (IR) cells were evident in the mucosal epithelium of both duodenum and jejunum (**Figure 15 A-C**). Double staining with chromogranin A (CgA) confirmed the endocrine identity of the CART IR cells (**Figure 15 A**). The majority (75±8% in duodenum and 54±15% in jejunum) of these cells were also GIP IR (**Figure 15 D**). Of all GIP IR cells, 20±7% (duodenum) and 8±3% (jejunum) were CART IR (**Figure 15 E**). A subset of the CART IR cells was also GLP-1 IR (4±4% in duodenum, **Figure 15 F** and <1%, in jejunum, data not shown). Of all GLP-1 IR cells, 16±16% in duodenum, **Figure 15 G** and <1%, in jejunum were CART IR. Confocal microscopy revealed colocalization of CART/GIP and CART/GLP-1 in the same secretory granules (data not shown), indicating that CART may be co-secreted with both GIP and GLP-1. Since co-expression of GLP-1 and GIP has been reported [142],

we triple-stained for CART, GLP-1 and GIP. This revealed that CART/GIP and CART/GLP-1 cells represent separate cell populations (data not shown).

Since CART was found to be expressed in enteroendocrine cells, we hypothesized that CART is released into the circulation after a meal. To test this, we performed OGTT (n=8) and MMT (n=9) in lean healthy volunteers and measured CART plasma levels.
Mean fasting CART plasma levels were 265±46 pmol/l (**Figure 16 A** and **C**). CART plasma levels displayed large individual variation and fluctuated between 28 and 875 pmol/l during the OGTT (**Figure 16 A-B**) and between 49 and 293 pmol/l during the MMT (**Figure 16 D-E**) without any apparent relation to meal or glucose ingestion. These observations agree with a previous report showing that circulating CART levels are unaffected by food intake [214]. However, when we normalized the CART concentrations in the postprandial samples for basal levels, a small increase in CART levels was seen 60 min after MMT (**Figure 16 F**). It should be noted that the measurements of CART were done in peripheral systemic blood. Since there are multiple sources of CART that may contribute to the circulating CART levels e.g. adrenal glands, pituitary, GI-tract, and pancreas [186], it would have been ideal to sample blood directly from the veins draining the intestine, to more directly assess whether intestinal CART is released into the circulation. This is ethically challenging to perform in humans. A previous study used such an approach in a pig model [267], however the pig enteroendocrine cells are devoid of CART [195]. Nevertheless, I can conclude that intestinal CART is most likely not released in high quantities into the circulation postprandially.

To further study the potential biological significance of K- and L-cell CART, we also studied how L- and K-cell CART is regulated by known stimulators of incretin expression and secretion *i.e.* glucose, palmitate and oleate [31] in GLUTag and STC-1 cells; model systems for L- and K-cells respectively [32, 33].
While GLP-1 had no effect on CART mRNA expression in GLUTag or STC-1 cells (**Figure 17 A** and **E** respectively), GIP increased CART mRNA levels in both GLUTag cells, (**Figure 17 A**) and STC-1 cells (**Figure 17 E**). Whether CART is secreted as a consequence of elevated GIP levels remains to be established. Assessment of CART secretion into the cell media is difficult due to the rather low CART expression in the

cells and not enough sensitivity of the available CART assays, thus the use of *in vivo* models would be preferable.

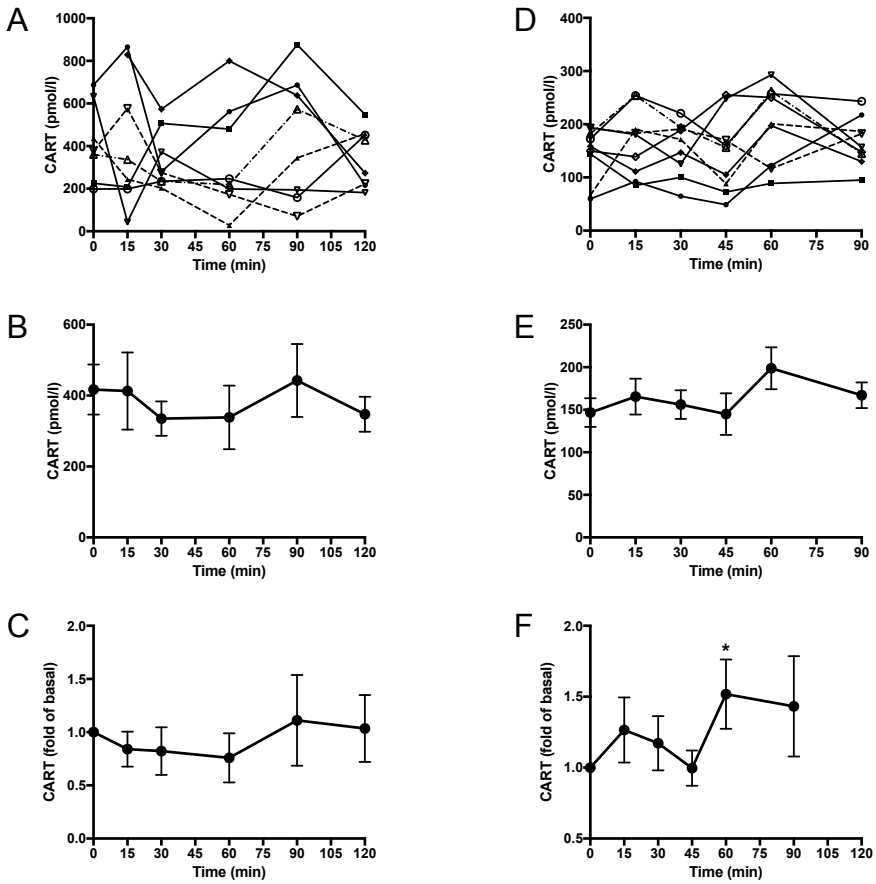


Figure 16. **CART plasma levels after an oral glucose load or a mixed-meal *in vivo* in humans**
CART plasma levels display large individual variation and seem unaffected by ingestion of 75 g glucose (**A-C**) (n=8) but are increased 60 min after a mixed-meal (**D-F**) (n=9). Each line indicates one test subject (**A and D**); data presented as means±SEM of the CART levels (pmol/l) (**B and E**); CART levels normalized to the basal CART concentration and presented as fold of basal (**C** and **F**), mean±SEM. *, $p < 0.05$.

In GLUTag cells, CART mRNA expression was unaffected by 24-h culture in 1-25 mM glucose (**Figure 17 B**) or oleate (**Figure 17 D**). Palmitate did not affect CART mRNA levels at 5.6 mM glucose, but increased CART mRNA levels at 25 mM glucose compared with cells cultured at 25 mM glucose (**Figure 17 C**).

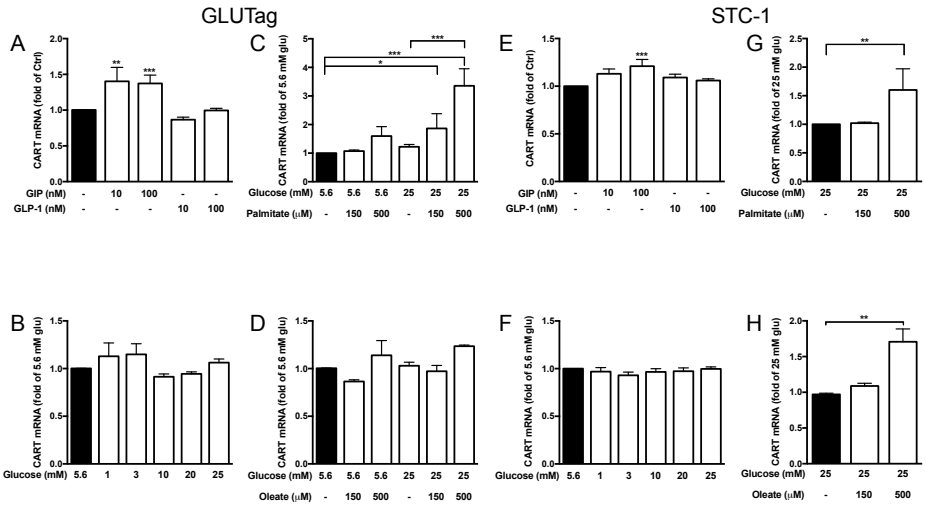


Figure 17. Regulation of CART mRNA expression in GLUTag cells and STC-1 cells GLUTag cells (**A-D**) and STC-1 (**E-H)** cells. GIP, but not GLP-1 increases CART mRNA expression in GLUTag cells (**A**) (n=3-9). CART mRNA expression is unaffected by 24-h culture in different concentrations of glucose in GLUTag cells (**B**) (n=4). At 25 mM glucose palmitate (**C**), but not oleate (**D**) increases CART mRNA levels in GLUTag cells **(**n=3-6). In STC-1 cells, GIP, but not GLP-1 increases CART mRNA levels (**E**) (n=3-7). In STC-1 cells CART mRNA expression is unaffected by 24-h culture in different concentrations of glucose (**F**) (n=4), but palmitate (**G**) and oleate (**H**) increase CART mRNA levels **(**n=3 and n=4 respectively). Data presented as mean±SEM. *, p <0.05; **, p<0.01; ***, p<0.001.

In STC-1 cells, CART mRNA expression was also unaffected by glucose (**Figure 17 F**), but both palmitate (**Figure 17 G**) and oleate (**Figure 17 H**). increased CART mRNA levels.

The finding that CART mRNA expression in GLUTag and STC-1 cells was inert to ambient glucose concentration is different from the situation in rat pancreatic beta-cells, in which CART expression is increased by glucose [189, 211, 268]. On the other hand, CART expression was increased after culture with palmitate or oleate, depending on cell type. The doses of FFAs were at the higher end and the possibility that CART expression is increased, as part of a stress response cannot be excluded.

Aim V. The role of CART in regulation of incretin secretion and expression

Having established expression of CART in K- and L-cells, we next aimed to understand the role of CART in these cells. We used STC-1 cells as a K-cell model and GLUTag cells as a L-cell model and also assessed the effects of CART administration *in vivo* in mice.
First, we assessed whether CART is a regulator of GIP and GLP-1 secretion and expression *in vitro*. To this end, we used treatment with exogenous CART peptide or silenced CART expression using siRNA in GLUTag and STC-1 cells.

Exogenous CART peptide (10 nM and 100 nM for 24 h) had no effect on GIP or GLP-1 mRNA expression in GLUTag (**Figure 18 A**) or in STC-1 cells (**Figure 18 B**). Furthermore, addition of exogenous CART (10 nM) to GLUTag cells during a 2-h GLP-1 secretion assay did not affect GLP-1 secretion at 0 or 16.7 mM glucose (**Figure 18 C**). I am confident that the CART peptide used in our study was biologically active, since peptide from the same batch and used at the same doses increased insulin secretion in parallel experiments [211].

CART KD in GLUTag cells resulted in the 82.0±0.9% reduction of CART mRNA (**Figure 18 D**), which led to a decrease in both GLP-1 mRNA expression and GLP-1 content (**Figure 18 D** and **E**). Next, active GLP-1 secretion was assessed during 2-h static incubations 72 h after CART KD in GLUTag cells. CART KD had no effect on GLP-1 secretion at 0 mM glucose. However, CART-silenced cells secreted 51.1±15.7% less GLP-1 at 16.7 mM glucose compared with control cells (**Figure 18 F**).
In STC-1 cells, CART KD led to a 62.0±3.4% reduction of CART mRNA (**Figure 18 G**). CART KD caused an approximately 1.2-fold increase in GIP mRNA expression (**Figure 18 G**) however GIP content was not affected by CART silencing (**Figure 18 H**). We were unable to assess the effect of CART silencing on GIP secretion due to GIP levels in the medium of STC-1 cells being below the detection limit of the ELISA. Thus, our results suggest that whereas exogenous CART does not affect GLP-1 expression or secretion, endogenous L-cell CART exerts stimulatory effects on GLP-1 expression and secretion. The exact mechanisms behind the effect are hard to dissect due to the lack of identified CART receptors [191, 238]. However, the present GLP-1 data are reminiscent of those obtained for insulin in beta- cells, in which

silencing of CART caused reduced insulin secretion and production [36] (Paper I). The observed effects on insulin were likely related to the reduced transcription of genes encoding proteins in the exocytotic machinery, as well as a network of beta-cell transcription factors, including *Tcf7l2* and *Isl1* both of which have been shown to regulate GLP-1 transcription in enteroendocrine cells [37-40]. Thus, even though further studies are needed to understand the mechanisms behind the effect of CART KD on GLP-1 secretion and expression, it is tempting to speculate that CART has similar roles also in GLUTag and SCT-cells.

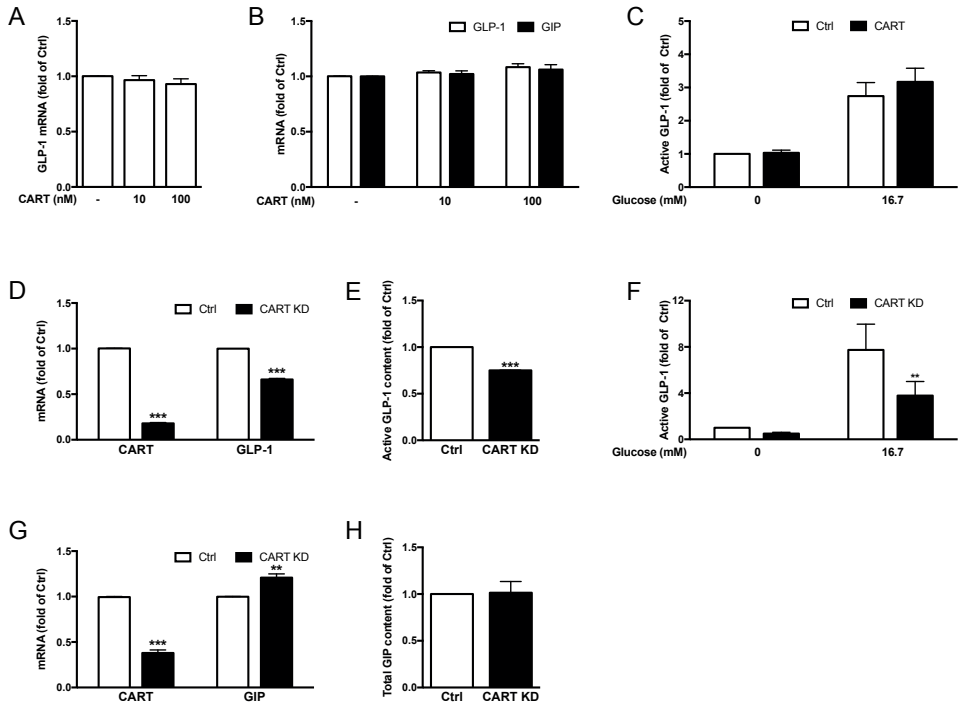


Figure 18. Effect of exogenous CART or CART silencing on incretin gene expression and GLP-1 secretion

Addition of exogenous CART does not affect incretin gene expression in GLUTag (**A**) or STC-1 (**B**) cells, and is without effect on glucose-stimulated GLP-1 secretion in GLUTag cells (**C**). CART silencing decreases GLP-1 mRNA expression (**D**) (n=3) and GLP-1 protein content in GLUTag cells (**E**) (n=4). CART silencing reduces GLP-1 secretion in response to glucose in GLUTag cells (**F**) (n=4). CART silencing increased GIP mRNA expression (**G**) (n=5), but was without effect on GIP protein content in STC-1 cells (**H**) (n=4). Data presented as mean±SEM. **, $p<0.01$; ***, $p<0.001$. Ctrl = scrambled siRNA; CART KD = CART siRNA.

Next, we tested the acute effect of exogenous CART on GIP and GLP-1 secretion *in vivo*. To this end, female C57Bl/6J mice (n=24 for total GIP secretion, n=21 for active GLP-1 secretion) were given CART peptide (150nmol/kg) intravenously (*i.v.*) during an OGTT. CART administration caused 1.5±0.2-fold elevated glucose-stimulated GIP secretion at 20 min (**Figure 19 A**), as well as 1.5±0.2-fold increased glucose-stimulated GLP-1 secretion at 10 min (**Figure 19 B**). Thus, our results suggest that administration of exogenous CART elevates GIP and GLP-1 secretion *in vivo*. On the other hand, we were unable to detect any direct effect of exogenous CART on GIP and GLP-1 mRNA expression and GLP-1 secretion *in vitro*. At this point, there is no clear explanation for this discrepancy. Although the doses of CART used here have been shown to stimulate GSIS [211], we cannot exclude that other doses of CART could affect GLP-1 and GIP secretion or expression in the cell lines employed. One potential explanation, could be that the stimulatory effect of CART on GIP and GLP-1 seen *in vivo* is indirect, e.g. via another hormone or via the enteric or central nervous system.

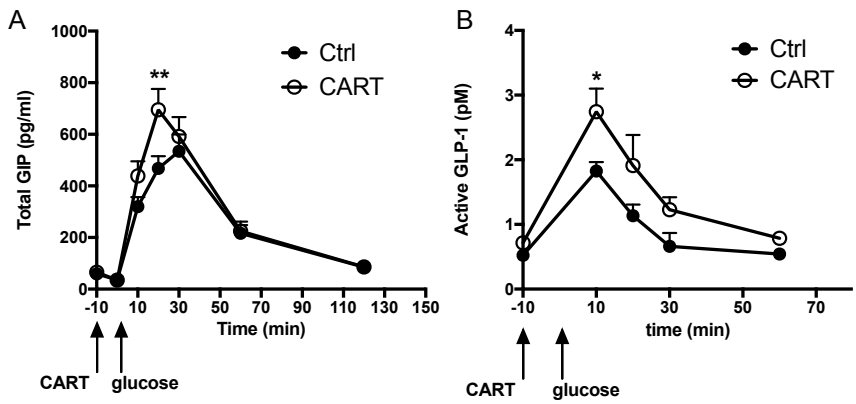


Figure 19. CART increases GIP and GLP-1 secretion during an OGTT in mice Intravenous administration of CART caused increased circulating levels of GIP (**A**) and GLP-1 (**B**) during an OGTT (n=24 and n=21 respectively) in mice. CART was given in a tail vein at -10 min and glucose orally at 0 min as indicated by arrows. Data presented as mean+SEM. *, p <0.05; **, p<0.01.

Studies in CART KO mice

Having established a regulatory role of CART for incretin secretion and expression, we next aimed to address the central question: is CART a hitherto unidentified incretin hormone? If so, CART could provide explanation for the incretin effect still present in double incretin receptor

KO mice [128, 129]. A key experiment addressing a potential role for CART as a novel incretin hormone would be to examine the incretin effect in CART KO mice.
The CART KO strain [198] previously available in our lab was terminated about 10 years ago due to an infection in the animal house. Furthermore, it turned out that there most likely were no living CART KO mice available on the globe. After discussions with Eli Lilly Inc. we were promised embryos of another strain [269] than the one previously used in our lab. First it took one year to get a material transfer agreement in place, thereafter it became clear after months of genotyping optimization that we were sent embryos of the wrong strain twice. Nonetheless, three years after since we started the process we had CART KO mice ready for experiments.
First, we performed OGTTs to assess the postprandial response in GIP and GLP-1 response. However, both female and male CART KO mice had identical GIP and GLP-1 levels as wild type controls. To assess whether there was a deranged incretin effect, we calculated the ratio of insulin secreted during intravenous glucose tolerance test (IVGTT) and OGTT. Also for this parameter, CART KO mice were indistinguishable from the wt controls. Furthermore, careful assessment of K- and L-cell density and incretin gene and protein expression in all relevant parts of the intestine (duodenum, jejunum, ileum and colon) failed to identify any differences between the genotypes.

Thus, the observed normal incretin effect and incretin secretion and production seen in CART KO mice, together with our data on only a minor increase in CART secretion in response to a meal in humans form the basis for my conclusion that CART is most likely not an incretin hormone.
It needs to be mentioned that the CART KO mice used had a global, whole body disruption of CART. It cannot be excluded that the lack of phenotype is related to redundant mechanisms. Ideally, an intestine-specific e.g. Villin-cre driven and inducible CART KO would have been preferable.

Summary

In this thesis, I have investigated the role of CART in beta-, K-, and L-cells. The findings on the role of CART in the regulation of insulin and incretin secretion and expression have led to increased understanding of regulation of beta-cell function and incretin biology. The knowledge obtained may pave the way for future treatment strategies for T2D. Conclusions from the presented studies are:

- CART regulates insulin production and secretion in beta-cells
- CART regulates ATP levels and beta-cell exocytosis of ready releasable pool granules
- CART regulates transcription of genes with key roles in beta-cell function including exocytotic genes, beta-cell transcription factors and genes important for insulin processing and secretion
- SCRT1 is a novel CART-regulated beta-cell transcriptional regulator
- SCRT1 regulates insulin synthesis, insulin secretion and exocytosis in beta-cells
- *SCRT1* expression is regulated by glucose in beta-cells and human islets
- SCRT1 is translocated from the nucleus to the cytosol in diabetic DEX rats
- CART is expressed in subpopulations of human K- and L-cells in the human upper small intestine
- CART plasma levels are increased after a mixed-meal in humans
- CART expression is regulated by GIP, palmitate and oleate *in vitro*
- CART KD in GLUTag cells reduces the expression and secretion of GLP-1
- Acute CART administration increases glucose-stimulated GIP and GLP-1 secretion during an OGTT in mice

Concluding Remarks and Future Perspectives

Previous studies have shown that stimulation with exogenous CART affects beta-cell function, and that absence of CART in the whole body results in reduced insulin secretion. The role, however, of CART produced within the beta-cell has remained unknown. My studies show that endogenous beta-cell CART is a regulator of insulin secretion at several different levels: it modulates ATP levels, insulin granule exocytosis, insulin transcription, insulin processing and glucose sensing. I have also linked CART to the regulation of a beta-cell transcriptional network important for beta-cell function and differentiation. This suggests that CART is important for maintaining the beta-cell fate. Whether inhibition of CART leads to transdifferentiation or dedifferentiation of the beta-cell is not known. This possibility may be first tested *in vitro* using MIN6 cells to distinguish cell-autonomous effects of transdifferentiation or dedifferentiation. Effects that require paracrine and endocrine signaling in an islet microenvironment then may be established using Ins1(Cre) and Ins1(Cre-estrogen receptor fusion protein) for beta-cell specific and time-specific CART inactivation or overexpression.

The finding that CART affected expression of transcription factors is in accordance with that CART was found to regulate thousands of genes including exocytotic genes, beta-cell transcription factors and genes important for insulin processing and secretion. This suggests an important regulatory role for CART in the beta-cells. The exact mechanisms underlying the transcriptional effects of CART remain to be elucidated. The possibility that CART in fact could be a transcription factor could be assessed using ChIP assays. Another possibility is that CART regulates gene expression via epigenetic modifications, such as altering DNA methylation or histone modification in the cells. Potential effects of CART on DNA methylation may be addressed using bisulfite conversion followed by qPCR or sequencing; alternatively, methylated DNA immunoprecipitation may be employed. Histone and DNA interactions

after CART gene targeting may be evaluated using ChIP followed by qPCR or sequencing or using chromatin accessibility assays. Guided by the RNAseq data on CART-silenced INS- (832/13) cells, I found SCRT1 to be a novel CART-regulated transcriptional regulator. SCRT1 expression was found in rodent and human beta-cells and my data show that SCRT1 is a regulator of insulin secretion and expression. Whether this is a direct effect is not known, but our preliminary ChIP data show that SCRT1 binds to the E-boxes in the *Ins1* promoter and affects genes with key roles in beta-cell function. Whether SCRT1 is mediating the effects of CART on transcription is not known and needs further investigation. Establishment of whether normalization of SCRT1 expression after CART KD rescues impaired beta-cell function would provide insight into it.

In view of the previously shown upregulation of CART in T2D beta-cells, my findings suggest that the upregulation of CART may serve as a compensatory mechanism to increase insulin secretion to sustain normal blood glucose levels. Whether the data obtained in rat cells translate into human beta-cell biology needs further investigation, but my data showing that *CARTPT* expression correlates with the expression of insulin, beta-cell transcription factors and exocytosis genes in human islets suggest that CART may be of importance also in human beta-cells. Functional gene targeting studies in human islets are warranted for elucidating the mechanisms of CART action in human beta-cells.

Furthermore, I have discovered a new site of CART expression in humans, *i.e.* the K-cells and L-cells in the upper small intestine. Reminiscent of the role of CART in beta-cells, endogenous L-cell CART was shown to exert a stimulatory action on GLP-1 secretion and expression. In K-cells on the other hand, CART seems to exhibit an inhibitory action on GIP transcription. The reason for the divergent effects is yet to be explained, but could potentially be related to different effects of CART on transcription factor expression or alternatively the effect is secondary to an effect of CART on other hormones expressed in the polyhormonal STC-1 cells. Future studies in primary intestinal cultures and single-cell RNA-sequencing of K- and L-cells would provide more insight into the mechanisms behind the effects of CART in the incretin-producing cells.

CART expression was shown to be regulated by free fatty acids, but not by glucose in K- and L-cell models. This resembles the situation *in vivo* in humans where CART secretion was stimulated by a fat-containing mixed-

meal, but not by a glucose load. Additionally, CART was found to be a stimulator of both GIP an GLP-1 secretion *in vivo* in mice. Whether fat-stimulated CART secretion contributes to the postprandial increase in GLP-1, GIP or insulin is not known, but my studies in mice with a global deletion of CART do not speak in favor of such an effect. However, redundant mechanisms may underlie the lack of phenotype; generation of more specifically targeted mice would better address the question. The finding of secretion of CART in response to a meal and insulinotropic properties of CART lead to the question if CART is a novel incretin hormone. However, even though we have no information on CART levels locally in the veins draining the intestine, the postprandial increase in CART plasma levels was rather small compared with the postprandial increase in levels of GIP and GLP-1. A possible strategy to further test whether CART is an incretin could be generation of mice with intestine-specific targeting of CART as well as immune-absorption of CART as was done for GIP in rat studies [157].

The available information positions CART as an anti-diabetic hormone. Apart from being a well-established inhibitor of appetite and food intake, CART has glucose-dependent insulinotropic and glucagonostatic properties, and CART protects beta-cells from glucotoxicity-induced cell death while reduced CART expression leads to increased beta-cell apoptosis. In addition, exogenous CART increases glucose-stimulated GIP and GLP-1 secretion and potentiates the insulinotropic effect of GLP-1. Furthermore, CART regulates adipocyte metabolism in insulin-like or insulin-antagonistic fashion depending on the cell milieu and preliminary data suggest that CART promotes GLUT4 translocation to the plasma membrane in muscle cells.

The above listed properties of CART largely resemble those of GLP-1 and imply a potential use of CART-based agents for T2D treatment.

While the role of CART in glucose homeostasis is rather well characterized, the exact mechanisms behind the observed effects are difficult to dissect due to lack of a characterized CART receptor(s). Our group is currently evaluating a potential receptor candidate identified in INS-1 (832/13) cells. The results are promising and we are intending to study whether the receptor mediates the effects of CART also in other cell types than beta-cells. The identification of the receptor would enable receptor-mediated disruption of CART signaling and development of

receptor agonists/antagonists. This will pave the way for better understanding of CART function in beta-cells, as well as other cell systems. It is also an important step towards evaluation of the therapeutic potential of CART-based therapy for treatment of T2D.

Thus, although the exact mechanisms of the CART-induced effects remain to be elucidated, I conclude that CART has an important role in the regulation of beta-, K- and L-cell function.

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Paper I

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Endogenous beta-cell CART regulates insulin secretion and transcription of beta-cell genes

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ABSTRACT

Impaired beta-cell function is key to the development of type 2 diabetes. Cocaine- and amphetamine-regulated transcript (CART) is an islet peptide with insulinotropic and glucagonostatic properties. Here we studied the role of endogenous CART in beta-cell function. CART silencing in INS-1 (832/13) beta-cells reduced insulin secretion and production, ATP levels and beta-cell exocytosis. This was substantiated by reduced expression of several exocytosis genes, as well as reduced expression of genes important for insulin secretion and processing. In addition, CART silencing reduced the expression of a network of transcription factors essential for beta-cell function. Moreover, in RNAseq data from human islet donors, *CARTPT* expression levels correlated with insulin, exocytosis genes and key beta-cell transcription factors. Thus, endogenous beta-cell CART regulates insulin expression and secretion in INS-1 (832/13) cells, via actions on the exocytotic machinery and a network of beta-cell transcription factors. We conclude that CART is important for maintaining the beta-cell phenotype.

1. Introduction

Type 2 diabetes (T2D) is a metabolic disease characterized by insufficient insulin secretion and insulin resistance (American Diabetes A, 2006; Marchetti et al., 2008). Impaired insulin secretion is key to the development of T2D (Ferrannini and Mari, 2004; Kahn, 2003; Levy et al., 1998). Therefore, improved knowledge about the mechanisms controlling beta-cell function is important for better understanding of T2D pathogenesis and for development of new treatment strategies. Insulin expression is regulated by a network of transcription factors (Melloul et al., 2002), including PDX-1, MAFA and NEUROD1 that bind to the insulin promotor region and are crucial for appropriate insulin synthesis and secretion, as well as for beta-cell survival (Zhang et al., 2005; Huang et al., 2002; Chu et al., 2001; Jonsson et al., 1994; Brissova et al., 2002; Ahlgren et al., 1998). NKX2.2 and NKX6.1 are additional beta-cell enriched transcription factors with crucial roles for beta-cell development and differentiation, regulating beta-cell maturation and expansion (Cerf, 2006; Sussel et al., 1998; Sander et al., 2000; Schisler et al., 2008; Taylor et al., 2013; Doyle and Sussel, 2007). Furthermore, it was recently shown that TCF7L2, through binding to ISL-1, regulates proinsulin production and processing via e.g. MAFA, PDX-1 and PCSK2 (Zhou et al., 2014).

Cocaine- and amphetamine-regulated transcript (CART) is a brain-gut peptide with anorexigenic properties (Ekblad et al., 2003; Lambert et al., 1998; Rogge et al., 2008). CART is also expressed in islet cells and in nerve terminals innervating the islets in several mammals, including humans (Ahrén et al., 2006; Abels et al., 2016; Wierup and Sundler, 2006). Exogenously added CART has been shown to increase insulin secretion in a glucose-dependent fashion from rodent and human islets and *in vivo* in mice due to enhanced beta-cell exocytosis. Furthermore, administration of exogenous CART inhibits glucagon secretion in human and rodent islets as well as *in vivo* in mice due to reduced alpha-cell exocytosis (Abels et al., 2016). Furthermore, exogenous CART protects beta-cells against glucotoxicity-induced cell death *in vitro* in INS-1 (832/13) beta-cells

Abbreviations: alpha-KIC, alpha-ketoisocaproate; CART, cocaine- and amphetamine-regulated transcript; CARTPT, human gene encoding CART; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GSIS, glucose-stimulated insulin secretion; KD, knock-down; OM, oligomycin; RRP, ready releasable pool.

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and rat islets (Sathanoori et al., 2013). In addition, endogenous beta-cell CART is upregulated in T2D patients as well as in several rodent models of T2D (Abels et al., 2016; Wierup et al., 2006), likely as a homeostatic response attempting to overcome hyperglycemia. Importantly, CART is necessary for maintaining normal islet function since *Cart* -/- mice display impaired insulin secretion and glucose intolerance due to islet dysfunction (Wierup et al., 2005). Although, it has been established that CART has insulinotropic actions when administered exogenously and that *Cart* -/- mice have reduced insulin secretion, the function of endogenous beta-cell CART is not known.

Here we addressed this using siRNA silencing of CART in INS-1 (832/13) beta-cells. Our data point towards an important role of CART as a regulator of insulin secretion by acting at multiple levels. CART silencing in INS-1 (832/13) cells resulted in decreased insulin secretion and insulin expression, reduced intracellular ATP levels and exocytosis. The observed effects were paralleled by reduced expression of beta-cell transcription factors and exocytosis genes. In addition, *CARTPT* expression correlated with *INS*, *MAFA*, *TCF7L2, SYT3* and *RAB3A* in human islets.

2. Materials and methods

2.1. INS-1 (832/13) beta-cell culture

INS-1 (832/13) cells (Hohmeier et al., 2000) were cultured in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) containing 2 g/l D-glucose, supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol (Sigma Aldrich). To study the effect of exogenous CART, CART 55–102 peptide (American Peptide Co Inc, Sunnyvale, CA, kind gift Prof. Michael J Kuhar, Emory University, Atlanta, GA, or Novo Nordisk A/S, Målöv, Denmark, kind gift Dr Lars Thim) was used.

2.2. siRNA-mediated gene silencing and qPCR

Gene silencing in INS-1 (832/13) cells was performed using Lipofectamin RNAiMAX (#13778150, Life Technologies, Waltham, MA) and 60 nM siRNA targeting rat *Cart* mRNA (#4390815, Silencer Select Pre-designed siRNA, Ambion, Life Technologies and J-090320-10-0002, ON-TARGETplus Rat Cartpt siRNA, Dharmacon, Lafayette, CO). The sequences for scrambled siRNA were sense: 5′-GAGACCCUAUCCGUGAUUAtt-3′ and antisense: 5′-UAAUCACGGAUAGGGUCUCtt-3′ (Silencer Select customer designed siRNA, Ambion, Life Technologies and D-001810-10-05, ON-TARGETplus Non-targeting control pool, Dharmacon). The transfection complexes were prepared according to the manufacturer's protocol. Total RNA was isolated 48 h after transfection and 1 μg of RNA was reverse-transcribed to cDNA using RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA). Quantitative RT-PCR was performed using the ABI Prism 7900 HT system (Life Technologies) with 15 ng cDNA and TaqMan gene expression assays (Life Technologies). All samples were analyzed with two endogenous controls (*Hprt* and *Ppia*). The gene expression levels were determined using the ΔΔCt method. TaqMan gene expression assays used were: *Cart* (Rn01645174_m1), *Tcf7l2* (Hs01009041_g1), *Mafa* (Rn00845206_s1), *Isl-1* (RN00569203_m1), *Pdx-1* (Rn00755591_m1), *Nkx6.1* (Rn01450076_m1), *Nkx2.2* (Rn04244749_m1), *NeuroD1* (Rn00824571_s1), *Ins1* (Rn02121433_g1), *Ins2* (Rn01774648_g1), *Hprt* (Rn01527840_m1), *Ppia* (Rn00690933_m1), *Stx1a* (Rn00587278_m1), *Syt4* (Rn01157571_m1), *Syt7* (Rn00572234_m1), *Syt13* (Rn00578161_m1), *Stxbp1* (Rn00564767_m1), *Snap25* (Rn00578534_m1), *Sytl4* (Rn00589676_m1), *Vamp2* (Rn00360268_g1), *Gck* (Rn00561265_m1), *Glut2* (Rn00563565_m1) (Life Technologies).

2.3. Immunoblotting of CART and proinsulin

INS-1 (832/13) cells were lysed 72 h after siRNA-mediated CART knock-down (KD) using Lysis-M reagent supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentration in the samples was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Life Technologies). The membranes were incubated overnight at 4 °C with primary antibody against CART (1:1000, code C4, Cocalico Biologicals, Reamstown, PA, kind gift Prof. Michael J Kuhar, Emory University, Atlanta, GA), proinsulin (1:1000, #8138S, Cell Signaling Technology, Beverly, MA) and β-actin (1:500, sc-47778 Santa Cruz Biotechnology, Dallas, TX). Detection was performed using horseradish peroxidase conjugated secondary antibodies and SuperSignal Femto Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Protein expression was quantified by band densitometry measurement using ImageJ software (Research Services Branch, National Institute of Health, Bethesda, MD).

2.4. Acid/ethanol extraction of insulin and proinsulin

INS-1 (832/13) cells were washed twice with PBS 72 h after siRNA-mediated CART KD, lysed in water and sonicated on ice. Thereafter hydrochloric acid/ethanol was added and samples were extracted at −20 °C for at least 24 h. Insulin and proinsulin content was determined using insulin/proinsulin ELISA (Mercodia, Uppsala, Sweden) and normalized to total protein content (Bio-Rad protein assay, Bio-Rad).

2.5. Glucose stimulated insulin secretion in INS-1 (832/13) cells

Glucose-stimulated insulin secretion (GSIS) was measured 72 h after CART KD. INS-1 (832/13) cells were washed twice and incubated for 2 h in 2.8 mM glucose HEPES-buffered saline solution (HBSS): 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, 0.2% BSA, pH 7.2, followed by 15-min- or 1 h stimulation in the same buffer containing 2.8 mM, 16.7 mM glucose, 2.8 mM glucose and 10 mM alpha-ketoisocaproate (KIC), 2.8 mM glucose and 35 mM KCl, 16.7 mM glucose and 4 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and 4 μg/ml oligomycin (OM), 16.7 mM glucose and 10 μM IBMX, and 16.7 mM glucose and 35 mM KCl. Insulin concentration in supernatants were determined using ELISA (Mercodia, Uppsala, Sweden) and were normalized to total protein content (determined by Bio-Rad protein assay, Bio-Rad) or to insulin content of each well as indicated.

2.6. Electrophysiology

Membrane currents and changes in membrane capacitance were evoked and recorded using an EPC10 amplifier and Patchmaster software (HEKA, Lambrecht/Pfalz, Germany) as described in (Salunkhe et al., 2016). Extracellular solutions contained: 118 mM NaCl, 20 mM TEACl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES, 5 mM Glucose (pH 7.4 with NaOH). Intracellular solution contained: 125 mM CsOH, 125 mM Glutamate, 10 mM CsCl, 10 mM NaCl, 1 mM $MgCl_2$, 3 mM Mg-ATP, 0.05 mM EGTA, 5 mM HEPES and 0.1 mM cAMP (pH 7.15 with CsOH). All experiments were performed at 33–34 °C.

2.7. ApoTox-Glo triplex assay

The ApoTox-Glo assay was used to assess cell viability,

cytotoxicity and apoptosis. CART KD was performed in INS-1 (832/13) cells and after 72 h ApoTox-Glo Triplex assay (Promega, Madison, WI) was used according to the manufacturer's instructions. Briefly, viability and cytotoxicity were measured by fluorescent signals produced when either live-cell or dead-cell proteases cleave added substrates (which have different excitation and emission spectra). Apoptosis was measured by the addition of caspase-3/7 substrate (Caspase-Glo 3/7, ApoTox-Glo Triplex Assay; Promega), which is cleaved in apoptotic cells to produce a luminescent signal.

2.8. ATP levels

To examine cellular ATP levels during GSIS, CART KD was performed in INS-1 (832/13) cells and 72 h later cells were washed twice and incubated for 2 h in 2.8 mM glucose HBSS. Thereafter the same buffer containing 2.8 mM, 16.7 mM glucose and 16.7 mM glucose with 4 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and 4 μg/ml oligomycin (OM) was added and cells were incubated for an additional 15 min. The Mitochondrial ToxGlo Assay (Promega) was then used according to the manufacturer's instructions to examine membrane integrity and cellular ATP levels. Briefly, protease activity associated with necrosis is measured first followed by ATP measurement after addition of ATP Detection reagent resulting in cell lysis and generation of luminescent signal proportional to ATP amount in the sample.

2.9. CARTPT expression in human islets

Expression of *CARTPT* was examined using RNA sequencing data on human islets from 195 cadaver donors provided by the Nordic Network for Clinical Islet Transplantation in Uppsala, Sweden and processed as previously described (Fadista et al., 2014). The correlation between *CARTPT* and beta-cell transcription factors and exocytosis genes in pancreatic islets was analyzed with Pearson's correlation coefficient and corrected for multiple testing (Bonferroni correction).

2.10. Statistics

Data were analyzed using one-way ANOVA or two-way ANOVA, followed by Bonferroni's test post hoc, or using unpaired Student's t-test. Protein expression was analyzed using Wilcoxon signed-rank test. Differences of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Silencing of CART reduces insulin secretion in INS-1 (832/13) cells

To investigate whether endogenous beta-cell CART regulates insulin secretion, we silenced *Cart* gene expression using siRNA in INS-1 (832/13) cells. This resulted in a 97.3 ± 0.5% reduction in *Cart* mRNA and 92.1 ± 9.9% reduction in CART protein levels ($p < 0.001$; Fig. 1A and B respectively). Raising the glucose concentration from

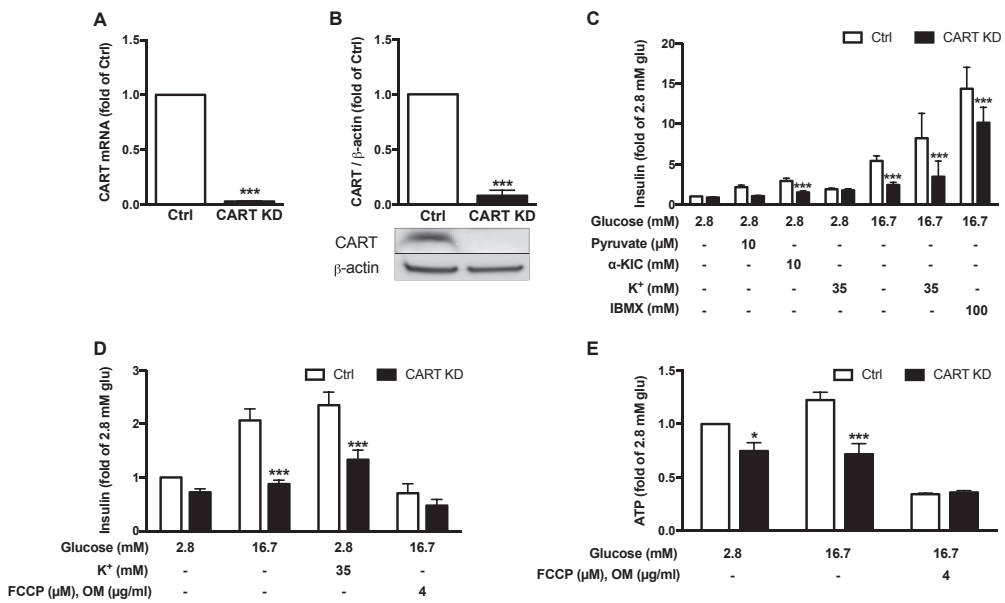


Fig. 1. CART KD reduces insulin secretion and ATP levels in beta-cells. A–B: Decreased *Cart* mRNA (A) and CART protein (B) expression after CART KD in INS-1 (832/13) cells relative to control (scrambled siRNA, Ctrl) (n = 4 and n = 6 respectively). C: CART KD reduces insulin secretion stimulated by 16.7 mM glucose, alpha-ketoisocaproate (alpha-KIC), K^+ (at 16.7, but not 2.8 mM glucose) and 3-isobutyl-1-methylxanthine (IBMX) during 1 h static incubations. D: During 15 min static incubations CART KD reduces insulin secretion stimulated by 16.7 mM glucose, as well as K^+-stimulated insulin secretion at 2.8 mM glucose. The effect was abolished by addition of the uncoupler carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) and the ATP synthase inhibitor oligomycin (OM) (n = 3–9). E: CART KD reduces ATP content at 2.8 and 16.7 mM glucose. The effect was abolished by addition of FCCP and OM (n = 3). Data presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$.

2.8 mM to 16.7 mM in control cells provoked a 5.4 ± 0.6-fold increase (Fig. 1C) in insulin secretion during the 1-h static incubation. Silencing of CART had no effect on basal insulin secretion (2.8 mM glucose), but caused reduced insulin secretion at 16.7 mM glucose (55.2 ± 6.0% reduction, $p < 0.001$; Fig. 1C). In addition, at 16.7 mM glucose CART KD reduced K^+ and IBMX-stimulated insulin secretion (58.2 ± 23.8% and 29.1 ± 13.4% reduction respectively, $p < 0.001$, Fig. 1C). At 2.8 mM glucose, CART KD had no effect on insulin secretion stimulated by K^+, but the response to alpha-ketoisocaproate (alpha-KIC) was reduced to 51.0 ± 6.9% ($p < 0.001$, Fig. 1C) and there was a trend towards decreased insulin secretion stimulated by pyruvate ($p = 0.07$, Fig. 1C). To study a potential effect of CART KD on first phase insulin secretion, we also assessed insulin secretion during 15-min static incubations. The stimulatory effect of K^+ at 2.8 mM glucose during 15-min stimulation was reduced in CART-silenced cells (2 ± 0.2-fold above basal for Ctrl vs 1.2 ± 0.1-fold for CART KD; $p < 0.001$, Fig. 1D). Reduced insulin secretion at 16.7 mM glucose after CART KD in INS-1 (832/13) cells was verified using another siRNA targeting *Cart* mRNA (Dharmacon, Lafayette, CO, 36.4 ± 13.4% reduction, $p < 0.001$, data not shown).

ATP is crucial for both the triggering and the amplifying pathways in glucose-stimulated insulin secretion (GSIS) (Wiederkehr and Wollheim, 2012). We therefore examined if CART KD affected ATP levels after 15-min incubation with glucose and inhibitors of mitochondrial ATP synthesis. Lower ATP levels were evident in CART KD cells at both 2.8 mM and 16.7 mM glucose (25.3 ± 7.9% lower at 2.8 mM glucose; $p < 0.05$ and 41.3 ± 8.1% lower at 16.7 mM glucose; $p < 0.001$, Fig. 1E). The reduced ATP levels were observed with only a very minor effect on protein levels in INS-1 cells after CART KD (7% lower in KD vs. Ctrl, $p = 0.01$, $n = 9$, data not shown). There was no effect of CART KD on ATP content after addition of the uncoupler FCCP and the ATP synthase inhibitor OM, suggesting that CART KD leads to a reduction in ATP synthesis by inhibiting mitochondrial metabolism.

3.2. Silencing of CART reduces insulin exocytosis in INS-1 (832/13) cells

As insulin secretion was reduced after CART KD in INS-1 (832/13) cells, and we recently showed that addition of exogenous CART increases beta-cell exocytosis in mouse islets (Abels et al., 2016), we assessed the effect of CART KD on exocytosis. To this end, we employed the standard whole-cell configuration of the patch-clamp technique. Exocytosis, measured as changes in membrane capacitance, was elicited by a train of ten 500-ms depolarizing pulses from −70 to 0 mV that evokes the influx of Ca^{2+} needed to stimulate fusion. While there was no significant change in the total increase in capacitance evoked by the train of depolarizations, CART KD caused a robust reduction in the capacitance increase evoked by the two first depolarizations (Fig. 2A–C). This is believed to represent exocytosis of primed granules in the readily releasable pool (RRP) of granules (Gillis et al., 1996; Olofsson et al., 2004). Thus, CART KD caused a reduction in the size of RRP from 133.4 ± 819.8 fF in Ctrl to 77.7 ± 7.7 fF in CART KD INS-1 (832/13) cells ($p < 0.05$; Fig. 2C). CART KD had no effect on charge or peak current, which corresponds to Ca^{2+} and Na^+ currents, respectively (Fig. 2D–E). In order to understand the basis for reduced exocytosis after CART KD, we next measured expression of genes important for exocytosis. Indeed, CART KD reduced expression of *Stxbp1*, *Syt7*, *Syt13*, *Sytl4*, *Snap25* and *Vamp2* (52.8 ± 1.7%, 88.5 ± 2.5%, 72.4 ± 2.9%, 38.8 ± 2.0%, 79.5 ± 2.7%, 72.0 ± 5.0% compared to control cells; $p < 0.01$, Fig. 2F), while *Stx1a* and *Syt4* expression was increased (120.4 ± 1.1%, 164.5 ± 2.5%, $p < 0.001$, Fig. 2F).

3.3. Silencing of CART in INS-1 (832/13) cells provokes decreased expression of insulin and genes involved in insulin secretion and production

We next addressed whether the reduced insulin secretion seen after CART KD could be explained by an impact on insulin

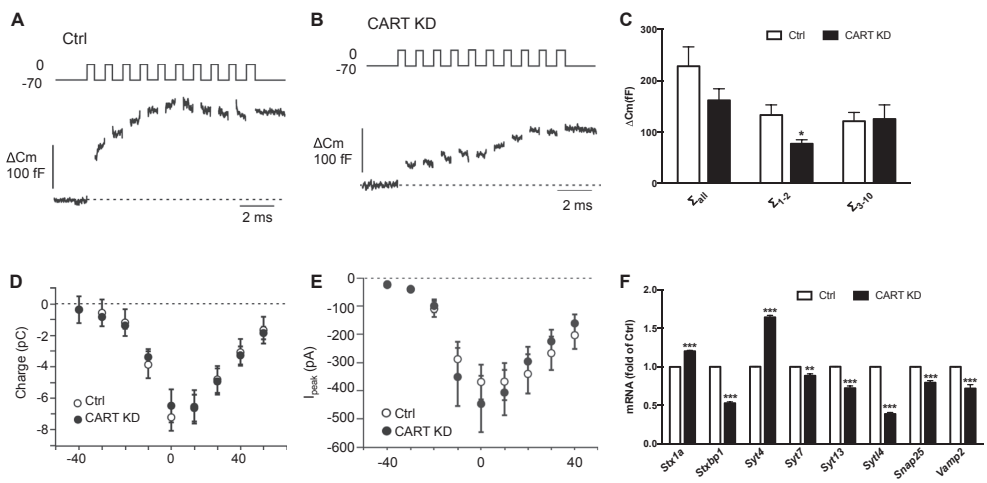


Fig. 2. CART KD reduces beta-cell exocytosis and alters expression of exocytosis genes. A–B: Representative traces of depolarization-induced exocytosis, measured as changes in cell membrane capacitance (ΔCm), in Ctrl (scrambled siRNA) (A) and CART KD (B) cells. C: Quantification of the capacitance changes induced by all depolarizations ($\sum_{all}$), by the first two depolarizations ($\sum_{1-2}$), corresponding to the readily releasable pool of granules, as well as by depolarizations 3–10 ($\sum_{3-10}$). D, E: Sustained charge (Q)–voltage (*V*) (D) and peak current (I_{peak})–voltage (*V*) (E) relationship measured in single Ctrl (scrambled siRNA) or CART KD cells. F: Effect of CART KD on expression of exocytosis genes. Data are expressed as mean ± SEM of 9–12 cells in each group (A–E) or 3 biological experiments (F). *$p < 0.05$, ***$p < 0.001$.

transcription and processing. CART KD in INS-1 (832/13) cells decreased *Ins1* and *Ins2* mRNA levels to 49.6 ± 3.7% and 55.7 ± 3.5% respectively ($p < 0.05$; Fig. 3A), that corresponded to 49 ± 7.0% decrease in proinsulin protein expression, as measured with Western blot ($p < 0.05$; Fig. 3B). Reduced proinsulin content (53 ± 6.3%, $p < 0.05$; Fig. 3C) and insulin content (75.1 ± 6.3%; $p < 0.05$, Fig. 3D) after CART KD were confirmed with ELISA. Reduced *Ins2* gene expression after CART KD in INS-1 (832/13) cells was verified using another siRNA targeting *Cart* mRNA (Dharmacon, 43.1 ± 3.3% reduction, $p < 0.01$, data not shown). To assess whether reduced insulin content could explain the reduction in insulin secretion seen after CART KD we repeated the 1 h insulin secretion experiments and normalized insulin secretion to insulin content of the cells. This revealed that CART KD reduced insulin secretion even when adjusted for insulin content (by 17 ± 6.8%; $p < 0.05$, Fig. 3E). We next examined the expression of beta-cell genes with important roles in insulin secretion/production. CART KD resulted in reduced mRNA levels of *Gck* (72.3 ± 4.2%; $p < 0.001$), *Glut2* (78.5 ± 3.4%; $p < 0.01$), *Pcsk2* (60.2 ± 10.1%; $p < 0.001$) and *Cpe* (63.5 ± 4.9%; $p < 0.001$) (Fig. 3F). However, *Pcsk1* mRNA levels increased to 136.4 ± 9.9% ($p < 0.001$, Fig. 3F).

3.4. Silencing of CART in INS-1 (832/13) cells represses key beta-cell transcription factors

Having established that CART affects expression of insulin and genes crucial for insulin secretion and processing, we next examined expression of the transcription factors known to regulate these genes (Melloul et al., 2002; Zhou et al., 2014; Fu et al., 2013). CART KD resulted in decreased expression of *Tcf7l2*, *Mafa*, *Isl-1*, *Pdx-1*, *NeuroD1*, *Nkx2.2* and *Nkx6.1* mRNA to 51.4 ± 3.7%, 39 ± 7.9%, 50.5 ± 4.3%, 61.7 ± 2.5%, 56.9 ± 5.9%, 65.1 ± 4.8%, and 79.7 ± 6.5% respectively ($p < 0.001$ and $p < 0.05$ for *Nkx6.1*, Fig. 3G). Reduced *Tcf7l2* and *Mafa* gene expression after CART KD in INS-1 (832/13) cells was verified using another siRNA targeting *Cart* mRNA (Dharmacon, 56.8 ± 8.8% reduction, $p < 0.001$ and 42.1 ± 15.9%

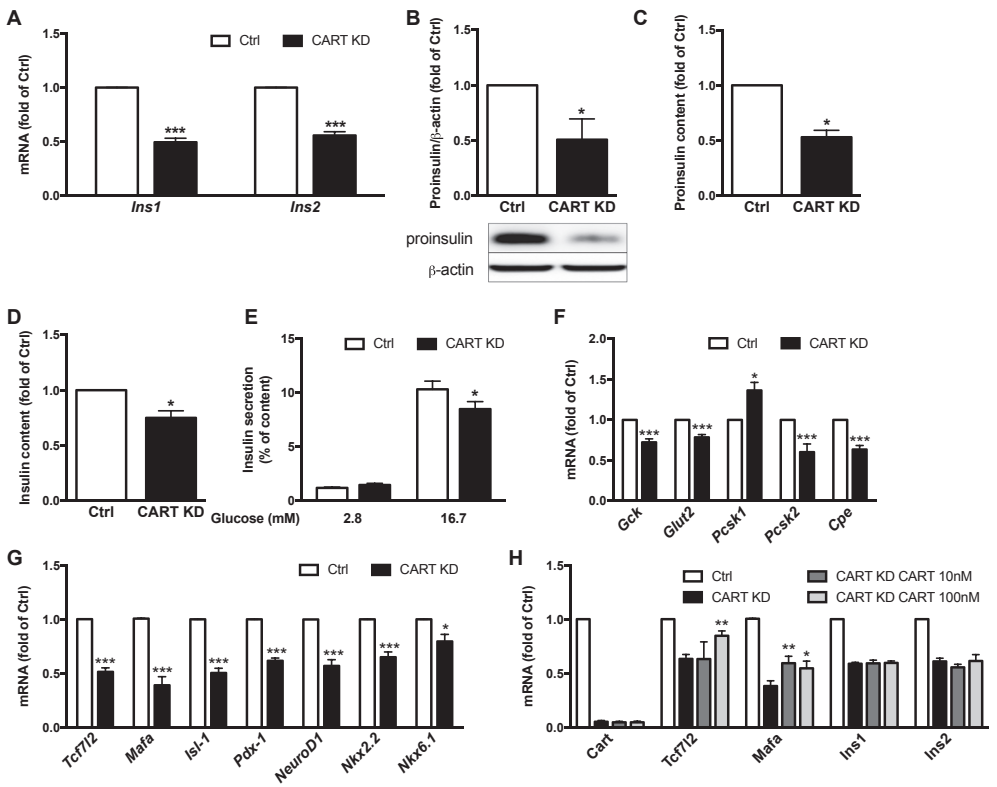


Fig. 3. CART KD decreases expression of insulin and important beta-cell genes. A: Reduced *Ins1* and *Ins2* gene expression after CART KD (n = 6) compared to Ctrl (scrambled siRNA). B: Band densitometry of Western blot and representative blot showing reduced proinsulin protein levels after CART KD (n = 7). C, D: Reduced proinsulin (C) and insulin (D) content after CART KD, measured using ELISA (n = 6). E: Insulin secretion (% of insulin content) at 2.8 mM and 16.7 mM glucose in Ctrl (scrambled siRNA) and CART KD (n = 6) as indicated. F: CART KD affects the expression of genes involved in glucose transport and sensing, as well as insulin processing (n = 4–6). G: Decreased *Tcf7l2*, *Mafa*, *Isl*-1, *Pdx*-1, *NeuroD1*, *Nkx2.2 and Nkx6.1* expression after CART KD (n = 3–6). H: 48 h CART treatment partially rescued expression of *Tcf7l2* and *Mafa*, but not insulin after CART KD (n = 2). Data presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

reduction, $p < 0.01$ respectively, data not shown). Finally we tested whether 48 h culture with exogenous CART could rescue the effect of CART KD. This revealed that addition of CART partially rescued expression of *Mafa* (increased by 21.3 ± 12%) and of *Tcf7l2* (increased by 16.4 ± 2%) after CART KD ($p < 0.01$) but was without effects on insulin expression (Fig. 3H).

3.5. CART silencing induces beta-cell apoptosis in INS-1 (832/13) cells

We have previously shown that addition of exogenous CART protects against glucotoxicity-induced beta-cell death (Sathanoori et al., 2013). To rule out that the observed effects of CART KD were secondary to reduced cell viability, we next assessed whether silencing of CART would influence beta-cell survival using the ApoTox-Glo Triplex assay. This method allows for simultaneous measurements of cell viability, cytotoxicity and apoptosis. CART KD caused an increase in caspase 3/7 activity (Fig. 4B), without affecting viability (Fig. 4A) or cytotoxicity (Fig. 4C).

3.6. The levels of CARTPT correlates with INS, TCF7L2, MAFA, SYT3 and RAB3A in human islets

We have previously shown a role for CART in human islet function. Specifically, addition of exogenous CART increased insulin secretion in human islets (Abels et al., 2016). To assess potential co-regulation of CART with genes important for beta-cell function, we performed linear regression analysis between the levels of *CARTPT* expression, and key beta-cell transcription factors, insulin and exocytosis genes using RNAseq data of human islets from 195 donors (Fig. 5) (Fadista et al., 2014).

The levels of *CARTPT* mRNA correlated with 23 of the selected genes (nominally significant, $p < 0.05$), of which 11 are genes involved in exocytosis (Suppl. Table 1). After Bonferroni correction for multiple comparison, the levels of *CARTPT* expression correlated positively with *INS* ($p = 0.001$, Fig. 5A), *MAFA* ($p = 0.025$, Fig. 5B), and exocytosis genes *SYT3* ($p = 0.007$, Fig. 5C) and small GTP-binding protein *RAB3A* that has been suggested to be involved in refilling the ready releasable pool of beta-cell granules (Yaekura et al., 2003; Regazzi et al., 1996; Iezzi et al., 1999) ($p = 0.009$, Fig. 5D), while the correlation between the levels of *CARTPT* and *TCF7L2* was negative ($p = 0.025$, Fig. 5E) in all subjects.

4. Discussion

CART is a novel constituent of human beta-cells and alpha-cells with insulinotropic and glucagonostatic effects when administered exogenously (Abels et al., 2016). Global *Cart*−/− mice have diminished insulin secretion (Wierup et al., 2005), but the underlying mechanisms for this has not yet been determined and the function of endogenous beta-cell CART has remained unknown.

Here we show that endogenous beta-cell CART plays an important role as a regulator of insulin secretion at multiple levels (summarized in Fig. 6). Thus, silencing of endogenous beta-cell CART leads to reduced insulin secretion by lowering ATP and attenuated exocytosis of docked and primed granules. In parallel, CART silencing decreased synthesis of insulin and expression of genes encoding proteins important for exocytosis, glucose sensing, and insulin processing, likely as a consequence of reduced expression of a network of transcription factors with key roles in beta-cell function.

Our present data on reduced insulin secretion in CART KD cells agrees with our previous data on glucose stimulated insulin secretion in *Cart* -/- mice (Wierup et al., 2005). Here, we stimulated insulin secretion with a battery of secretagogues to dissect the mechanistic basis for the effect of CART on insulin secretion. This revealed that CART KD reduced insulin secretion stimulated by glucose alone, cAMP, K^+, and the mitochondrial fuel alpha-KIC. This suggests that CART acts at late events of insulin secretion. In agreement, CART KD reduced exocytosis, and in particular exocytosis of granules from the RRP. This finding gains support from our previous observations that addition of exogenous CART increases beta-cell exocytosis in mouse islets (Abels et al., 2016). The finding that CART KD reduced K^+-stimulated insulin secretion (at 2.8 mM glucose) after 15 min, but not after 1 h is likely explained by that the effect of CART KD is primarily on primed granules. The fact that our patch-clamp experiments were performed in the presence of ATP and that CART KD only reduced primed granules, without reducing the Ca^{2+} current, suggests a direct effect on the exocytotic machinery (Eliasson et al., 1997). This is, most likely related to the observed reduced expression of key exocytosis genes, a notion supported by the fact that 24-h CART treatment was needed for stimulatory effect of exogenous CART on beta-cell exocytosis (Abels et al., 2016). Indeed, CART KD mediated downregulation of *Vamp2* and *Snap25,* whereas *Stx1a* expression was increased, implicating importance of CART in regulating expression of proteins involved in granular fusion (Eliasson, 2014). CART KD thus alters the stoichiometry of the SNARE proteins, which has been shown to cause inhibition of insulin secretion (42). SNAP25 has earlier been demonstrated to have a fundamental role in beta-cell exocytosis through mechanisms that are independent of the Ca^{2+}-influx (Vikman et al., 2006, 2009). Syntaxin 1 together with STXBP1 are critical for the formation of granular docking sites (Gandasi and Barg, 2014) and STXBP1 together with SYTL4 are important for docking (Tomas et al., 2008). Thus, the CART KD induced reduction of *Stxbp1* expression and increase in *Stx1a* and *Syt4* expression is more complex, but might explain why no effect is observed on granular docking and mobilization (represented by increase in

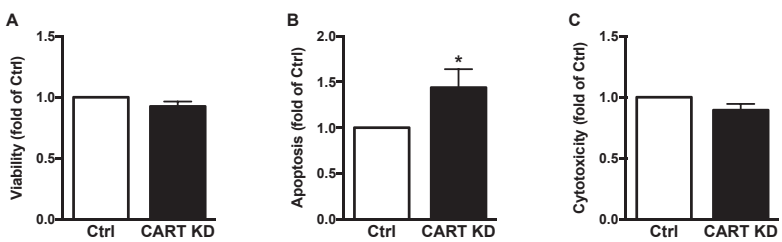
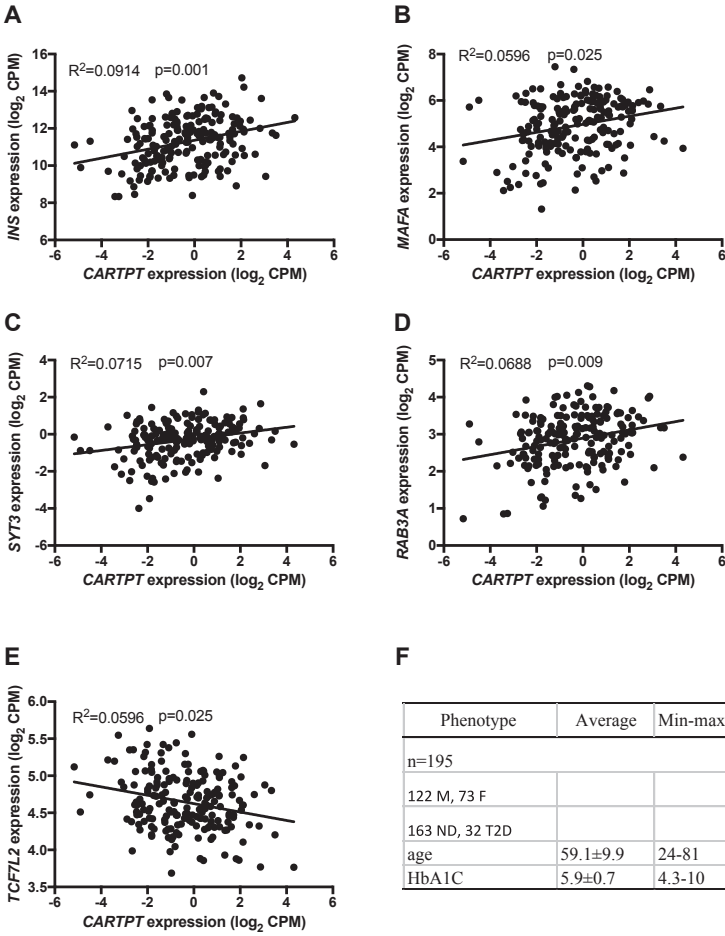


Fig. 4. CART KD increases beta cell apoptosis but does not alter viability or cytotoxicity. CART KD in INS-1 (832/13) cells results in increased apoptosis (B), without affecting cell viability (A) or cytotoxicity (C) ($n = 6$) compared to Ctrl (scrambled siRNA). Data presented as mean ± SEM. *$p < 0.05$.



F

Phenotype	Average	Min-max
n=195		
122 M, 73 F		
163 ND, 32 T2D		
age	59.1±9.9	24-81
HbA1C	5.9±0.7	4.3-10

Fig. 5. *CARTPT* mRNA correlates with important beta cell genes in human islets. *CARTPT* expression correlates positively with *INS* (A), *MAFA* (B), *SYT3* (C) and *RAB3A* (D), but negatively with *TCF7L2* (E) in RNAseq data from 195 human islet donors. F: Islet donor characteristics. T2D: type 2 diabetic donors; ND: non-diabetic donors; M: male; F: female.

membrane capacitance by the later depolarizations of the train). Interestingly, expression of the synaptotagmin genes *Syt7* and *Syt13* was slightly reduced after CART KD. Depletion of SYT7 in knock-out animals leads to reduced first phase insulin release (Gustavsson et al., 2008), and first phase insulin secretion has been suggested to be associated with the release of RRP granules (Eliasson et al., 1997), whereas not much is known about the function of SYT4 and SYT13 in beta-cell exocytosis. However, silencing of SYT13 in INS-1 (832/13) cells reduces glucose stimulated insulin secretion and the expression of SYT4, SYT7 and SYT13 is reduced in islets of T2D donors (Andersson et al., 2012).

In the present study, we also show that CART KD caused a reduction in ATP content, unlikely to be linked to decreased viability since total protein content was only marginally decreased after CART KD. This together with our present and previous findings that the effect of exogenous CART is glucose-dependent suggests a role for CART in glucose metabolism. We found that CART KD caused reduced expression of *Glut2* (although not rate-limiting for glucose metabolism) and *Gck*. This could in turn lead to less glucose available for the glycolysis and hence less pyruvate available for ATP production in the mitochondria. Reduced expression of GLUT2 was also evident in islets from *Cart* -/- mice (Wierup et al., 2005). On the other hand, the inhibitory effect of CART KD on GSIS was still present under stimulation with alpha-KIC indicating that CART may be important for oxidative phosphorylation. CART has been shown to preserve ATP levels after ischemic conditions via interaction with succinate dehydrogenase (SDH, complex II) in neurons (Mao et al., 2007). ATP is indeed an important trigger and amplifying factor of

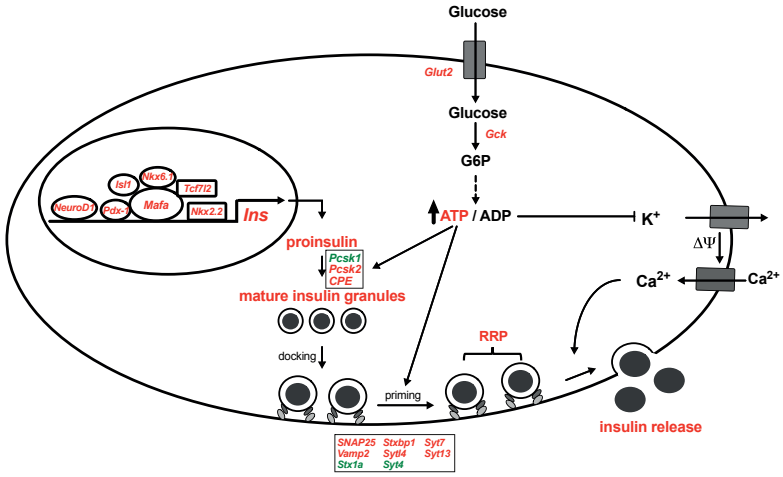


Fig. 6. Cartoon illustrating a model of how endogenous beta-cell CART regulates insulin secretion and production. Red indicates reduced, and green - increased expression or levels after CART KD.

beta-cell exocytosis (Eliasson et al., 1997; Ashcroft and Rorsman, 2013) and the effect of CART KD on insulin secretion was lost after uncoupling with FCCP and blocking ATP synthesis with OM. On the other hand, exocytosis was decreased during the patch clamp experiments performed in the presence of ATP, suggesting involvement of additional mechanisms to ATP in the reduced insulin secretion after CART KD.

Addition of exogenous CART has previously been shown to protect against glucotoxicity-induced cell death in rat islets and INS-1 (832/13) cells (Sathanoori et al., 2013). However, CART KD had no major effect on cell viability or cell death, although a moderate increase in apoptosis was observed. This is in line with our observations that *Cart* -/- mice have normal islet size (Wierup et al., 2005). We therefore feel confident to conclude that the observed effects of CART KD are not secondary to reduced cell viability.

We showed that CART KD caused reduced insulin synthesis. Thus, reduced insulin mRNA, proinsulin content and insulin content were evident after CART KD. We also provide evidence for that this reduction in insulin content does not alone explain the reduced GSIS seen after CART KD, since lower GSIS was evident even after normalizing to insulin content. Prohormone convertases 1/3 and 2 and carboxypeptidase E cleave proinsulin to insulin and C-peptide (Steiner et al., 2009). We found that CART KD decreased *Pcsk2* and *Cpe*, but on the other hand increased *Pcsk1*. This is an interesting finding that may have implications for processing of peptides differentially processed by these enzymes (e.g. the proglucagon peptide), but does not suggest a major role for CART as a regulator of processing of proinsulin to insulin. Rather we believe that the observed reduced insulin synthesis is a consequence of reduced expression of a network of key beta-cell transcription factors, in turn affecting insulin transcription. Thus, CART KD reduced expression of *Mafa*, *Pdx-1*, *Isl1*, *NeuroD1*, *Nkx2.2* and *Nkx6.1* that can activate insulin transcription or regulate beta-cell development and differentiation (American Diabetes A, 2006; Melloul et al., 2002; Chu et al., 2001; Brissova et al., 2002; Zhou et al., 2014; Ediger et al., 2014; Glick et al., 2000). CART KD also reduced *Tcf7l2*, the number one risk gene for T2D so far identified. Culture with exogenous CART was without on insulin expression, but partly rescued expression of *MafA* and *Tcf7l2* after CART KD. Whether a complete rescue can be achieved with other concentrations of CART and other culture times needs further investigation. TCF7L2 was recently shown to bind to ISL1 and controlling expression of PDX-1, NEUROD1, NKX6.1 and MAFA transcription factors, as well as other key beta-cell genes (including PCSK1 and PCSK2) controlling insulin processing, maturation and secretion (Zhou et al., 2014). In addition, decreased ATP content after CART KD could also contribute to the reduced insulin content (Orci et al., 1987; Rhodes et al., 1987). Although the exact mechanisms are difficult to dissect due to the lack of identified CART receptors (Rogge et al., 2008; Vicentic et al., 2006), our data position CART as an important regulator of beta-cell transcription.

Finally, we used RNAseq in human islets to assess whether our findings in INS-1 (832/13) cells have relevance in man. Indeed, the level of *CARTPT* expression was correlated with *INS*, *MAFA*, *TCF7L2*, *SYT3* and *RAB3A*. Together with our previous data showing that CART increases insulin secretion, reduces glucagon secretion and is expressed in human beta-cells as well as alpha-cells, and is upregulated in T2D islets (Abels et al., 2016), this suggests important roles for CART also in human islets.

In summary, our data point towards important roles for endogenous CART in the beta-cell since CART silencing resulted in reduced (1) insulin synthesis, (2) insulin secretion via reduced ATP and exocytosis of RRP granules, (3) expression of beta-cell key genes regulating transcription, glucose sensing, insulin processing and exocytosis.

5. Conclusions

We conclude that endogenous beta-cell CART plays important roles in regulation of beta-cell function and suggest that CART expression is necessary for maintaining the beta-cell phenotype.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2017.02.027.

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Paper II

Characterization of genetic networks regulated by CART in INS-1 (832/13) cells

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ABSTRACT

Cocaine- and amphetamine-regulated transcript (CART) has recently been described as a regulator of beta-cell function. Downregulation of CART in INS-1 (832/13) cells caused impaired insulin secretion and production, likely due to actions on the exocytotic machinery and a network of beta-cell transcription factors. CART has also been shown to protect beta-cells from glucotoxicity via reduced apoptosis and increased proliferation, and activation of key pro-survival pathways. To increase our understanding of the role of CART in beta-cells and to further characterize CART-activated genetic networks implicated in beta-cell function, we performed RNA-sequencing on CART-silenced INS-1 (832/13) cells. Differential expression analysis revealed that 5026 genes (25.5% of all identified genes) were differentially expressed; 2495 genes were downregulated and 2531 genes were upregulated. The most strongly upregulated genes were *AABR07068253.1*, *Jun* and *Srp14*; in contrast, *Adam11*, *Pak3* and *Ppp1r17* showed the most decreased expression after CART KD. The most significantly differentially expressed genes related to insulin secretion were *Stxa1*, *Gnas* and *Stxbp1,* and the genes related to insulin signaling that were most differentially expressed were – *Fasn*, *Crk*, *Ptpn1*. Among genes involved in cell cycle and apoptosis processes, CART KD most significantly affected expression of *Gadd45g*, *Crebp*, *Anapc* and *Nfkb1*, *Jun* and *Tuba1a*. Functional annotation clustering classified CART-regulated genes into 11 clusters which were downregulated and 10 which were upregulated. Energy derivation and cellular respiration, cell death, protein transport and mitochondrial genes were among the upregulated clusters while pathways related to response to carbohydrate stimulus, zinc ion binding, transcription regulation, as well as signal transduction were downregulated. We also mapped the differently expressed genes after CART KD into pathways using Ingenuity Pathway Analysis software. This revealed that 281 pathways were significantly affected by CART KD of which molecular mechanisms of cancer, PKA signaling and cardiac hypertrophy signaling were most significantly affected.

Insulin receptor signaling, AMPK signaling and T2D diabetes signaling were among the top 10% of the most regulated pathways. In the present study we focused on T2D signaling and Ingenuity Pathway analysis suggested that reactive oxygen species, KIR6.*2* and SUR1 and the beta-cell transcription factors *Pdx-1*, *Mafa* and *Cebpb* may mediate the effects of CART on insulin secretion and survival. CART KD was also predicted to inhibit glycogenesis, lipolysis and protein synthesis through the modulation of insulin signaling and to promote GLUT4 translocation to the plasma membrane through IKK, NF-kB, PPARγ-and IRS2.

In summary, we have characterized CART-activated genes and functional clusters in INS-1 (832/13) cells. CART was shown to affect the expression of genes with important roles for insulin secretion, insulin signaling, apoptosis and cell cycle regulation. We conclude that endogenous CART is important for beta-cell function.

Keywords: Cocaine- and amphetamine-regulated transcript; beta-cell; T2D; INS-1 (832/13)

INTRODUCTION

Type 2 Diabetes (T2D) is characterized by insufficient insulin secretion and insulin resistance [1, 2]. Impaired insulin secretion is key to the development of T2D [3-5] and therefore, increased knowledge about the regulation of beta-cell function is important to understand T2D pathogenesis and for developing new treatment strategies. Cocaine- and amphetamine-regulated transcript (CART) regulates several aspects of beta-cell function, including insulin granule exocytosis [6, 7], insulin expression [6] and beta-cell proliferation and apoptosis [8]. CART is expressed in both islet endocrine cells and in parasympathetic and sensory nerves innervating the islets. In the developing rat, islet CART is upregulated in all islet cell types, except the ghrelin cells. In developing mice, CART is mainly expressed in the alpha-cells. In the adult rat, the intra-islet expression of CART is limited to the somatostatin-producing delta-cells, while in adult mice CART is mainly expressed in nerve fibers but also in a small subpopulation of beta-cells [9]. In the human pancreas, CART is found in islet alpha- and beta-cells and in cholinergic nerve terminals innervating the islets [7, 10, 11]. Endogenous beta-cell CART has been reported to be upregulated in T2D patients and rodent models of T2D [7, 9]. Exogenous CART has been shown to stimulate insulin secretion and to potentiate the effect of glucagon-like peptide-1 (GLP-1) in INS-1 (832/13) cells and in rat islets *in vitro* [9] and *in vivo* in mice [7]. The insulinotropic effect *in vitro* was reported to be 3'-5'-cyclic adenosine monophosphate/protein kinase A (cAMP/PKA)-dependent [9]. In addition, under glucotoxic conditions exogenous CART protects the beta-cell through increased proliferation and reduced apoptosis via key regulators of cell survival and proliferation including CREB, IRS proteins, PKB, and p44/42 MAPK [8].

We have previously shown in INS-1 (832/13) cells that silencing of endogenous CART leads to impaired insulin secretion and expression [6]. CART KD resulted in decreased expression of *Tcf7l2*, *Mafa*, *Isl-1*, *Pdx-1, NeuroD1, Nkx2.2* and *Nkx6.1,* and reduced insulin secretion by lowering ATP and by attenuated exocytosis of docked and primed granules [6]. As CART KD decreased the expression of key beta-cell transcription factors and impaired beta-cell function,

we assumed the involvement of a number of additional genes important for the beta-cell phenotype in the observed effects in INS-1 (832/13) cells. In the present study, we aimed to understand the mechanistic basis for the effect of CART on beta-cell function by characterizing the effects of CART silencing on gene expression in INS-1 (832/13) cells using RNA-sequencing (RNAseq).

Differentially expressed gene analysis was used to identify potential CART-regulated pathways and to elucidate downstream effects of CART silencing.

2. MATERIALS AND METHODS

2.1. INS-1 (832/13) cell culture

INS-1 (832/13) cells were cultured in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) containing 2 g/l D-glucose, supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol (Sigma Aldrich).

2.2. siRNA-mediated gene silencing and RNA-sequencing

Gene silencing in INS-1 (832/13) cells was performed using Lipofectamin RNAiMAX (#13778150, Life Technologies, Waltham, MA) and 60 nM siRNA targeting rat *Cart* mRNA (s130729, Silencer Select Pre-designed siRNA, Ambion, Life Technologies). The sequences for scrambled siRNA were sense: 5'-GAGACCCUAUCCGUGAUUAtt-3' and antisense: 5'-UAAUCACGGAUAGGGUCUCtt-3' (Silencer Select customer designed siRNA, Ambion, Life Technologies). The transfection complexes were prepared according to the manufacturer's protocol. Total RNA was isolated 48 h after transfection and quality was assessed with the 2200 Tapestation (Agilent Technologies). One microgram of total RNA of sufficient quality (RIN > 8) was used for sample preparation for sequencing with a TruSeq RNA sample preparation kit (Illumina Inc., San Diego, CA).

Transcriptome sequencing was performed on the Illumina HiSeq 2000 platform using a paired-end 101 bp protocol (Illumina Inc., San Diego, CA). A library was constructed using Illumina TrueSeq RNA sample preparation kit, paired end (Illumina Inc.). Amplification was performed for 101 cycles. Six libraries were multiplexed on each line of the flowcell. The average number of paired-end reads for each sample obtained was 32 million reads. Reads were mapped by STAR v.2.4.1 to the rat reference, Rattus_norvegicus v. 6.0.83, downloaded from ENSEMBL.

2.3 Differential expression analysis

To identify genes that are affected by CART KD we followed the protocol described in DEseq2. Uniquely mapped reads were identified using FeatureCount with flags (-p -t exon -g gene_id) in the subread v.1.5.0 package. Normalization, using rlog, and differential expression analysis was carried out using DESeq2_1.14.1 package. For each gene a fold-change value and an adjusted p-value, using Benjamin-Hochberg correction for multiple testing, were reported.

2.4. Pathway analysis

To assess functional enrichment, genes with substantially changed expression (adjusted *p*-value of ≤0.05 for multiple comparisons) were subjected to gene ontology (GO) analysis using the functional annotation tool DAVID 6 [12, 13] to identify which biological processes are enriched within a list of genes. In addition, differentially expressed genes (adjusted *p*-value of ≤0.05) and their corresponding fold-change values were uploaded into the Ingenuity Pathway Analysis (IPA®) software (Qiagen, Redwood City, CA) for analysis. The identified canonical pathways were considered significant, and highly up- or downregulated, if $p < 0.05$.

3. RESULTS

3.1 Effects of CART silencing on gene expression in general

CART silencing resulted in a 5.4-fold decrease in CART transcript levels (**Table 1**). RNAseq analysis revealed that of all 19696 genes that had enough read coverage to be analyzed by DEseq2, 2495 genes were upregulated and 2531 genes were downregulated after CART silencing (adjusted *p*-value of ≤0.05 for multiple comparisons). The top 50 downregulated genes and the top 50 upregulated genes are presented in **Table 1A** and **B**, respectively. *AABR07068253.1* (similar to Signal Recognition Particle Alu Rna Binding Heterodimer, Srp914) was identified as the most strongly upregulated gene after CART KD. The heterodimeric protein complex SRP9/SRP14 (SRP9/14) is a component of the signal recognition particle (SRP) [14, 15] and is involved in the regulation of translation where it is required for elongation of polypeptide chains or possibly for inhibition of polysome formation and initiation of protein synthesis [16]. The Signal Recognition Particle 14, *Srp14* appeared as the third gene most upregulated by CART KD. *Jun* was the second most strongly upregulated gene after CART KD. It encodes the part of the AP-1 transcription factor complex that has been implicated in cell differentiation, proliferation and oncogenic transformation [17]. Decreased expression of APMAP (the fifth most upregulated gene) has been shown in adipose tissue of patients with gestational diabetes and it has been suggested to contribute to insulin resistance in adipose tissue [18]. The top ten upregulated genes also include *Sfta2* (Surfactant-Associated Protein 2), *F3* (Coagulation Factor III), *LOC100910404* (actin), *Ndnf* (neuron derived neurotrophic factor), *Ldhb* (Lactate Dehydrogenase B) and *Rhob* (Ras Homolog Family Member B).

The most strongly downregulated genes include gene-candidate tumor suppressor *Adam 11* [19], and *Pak3* (P21 (RAC1) Activated Kinase 3), a serine/threonine kinase of the PAK family that is important for synaptic plasticity in mice. Importantly, PAK3 has recently been shown to act

downstream of Ngn3 and to regulate beta-cell differentiation and function [20]. The top ten downregulated genes also include *Ppp1r17* (Protein Phosphatase 1 Regulatory Subunit 17/G-substrate), *Btbd17* (BTB Domain Containing 17), *Trim72* (Tripartite Motif Containing 72), *Igsf11* (Immunoglobulin Superfamily Member 11), *Lmbrd2* (LMBR1 Domain Containing 2), *Hpca* (Hippocalcin), *AC142182.1* and *Scrt1* (Scratch Family Transcriptional Repressor 1).

3.2 Changes in beta-cell related gene expression after CART silencing in INS-1 (832/13) cells

We next assessed genes with established importance for insulin secretion using a gene list acquired from Kyoto Encyclopedia of Genes and Genomes (KEGG) [21]. The top three differentially expressed genes related to insulin secretion and exocytosis (based on adjusted p-value) were *Stxa1*, *Gnas* and *Stxbp1* (**Table 2**) and to insulin signaling – *Fasn*, *Crk*, *Ptpn1* (**Table 3**). Genes related to insulin secretion/exocytosis significantly affected by CART KD also included other exocytotic genes including synaptotagmins, *Vamp2*, *Snap25*, *Rab3a*, beta-cell transcription factors e.g. *Pdx-1*, *Isl1*, *Neurod1*, *Nkx2.2*, as well as genes important for insulin processing e.g *Pcsk1*, *Pcsk2*, *Cpe* (**Table 2**). We also confirmed the altered expression of most of the previously [6] qPCR-identified CART-regulated genes (**Table 4**).

3.3 Changes in cell cycle and apoptosis gene expression after CART silencing in INS-1 (832/13) cells

We also assessed genes involved in the regulation of cell cycle and apoptosis as CART has been shown to have cytoprotective properties [8] and to be expressed in certain tumors [22, 23]. We found differential expression of 29 genes involved in cell cycle control (**Table 5**) and 35 genes involved in the regulation of apoptosis (**Table 6**). The top three differentially expressed cell cycle genes include *Gadd45g*, *Crebbp* and *Anapc1*, and the top three apoptosis genes were *Nfkb1*, *Jun* and *Tuba1a* (sorted based on adjusted p-value).

3.4 Functional clustering

The differentially expressed genes were further classified into functional clusters. Gene ontology (GO) analysis using DAVID 6 [12, 13] revealed 11 clusters that were downregulated and 10 clusters that were upregulated after CART KD (**Tables 7** and **8**). Thus, in addition to the previously described cellular functions of CART (e.g mitochondria, energy derivation, response to glucose stimulus and cell death [6, 8, 24, 25]), the analysis also suggested CART to be involved in the regulation of protein transport, nuclear lumen-ER network, macromolecule catabolic processes and cell junction.

3.5. Pathway analysis of differentially expressed genes after CART silencing in INS-1 (832/13 cells)

In order to further explore cellular functions potentially affected by CART silencing, we used the IPA software for Canonical Pathway Analysis. This revealed that CART KD affected 281 canonical pathways ($p<0.05$; **Table 9**). Molecular mechanisms of cancer, PKA signaling and cardiac hypertrophy signaling were the most significantly affected pathways———. Insulin receptor signaling, AMPK signaling and T2D diabetes signaling were among the top 10% most regulated pathways. We focused on the pathways of T2D signaling to gain insight into the role of CART in insulin secretion. The prediction tool of the IPA software suggested a glucotoxicity-mediated increase in ROS that via down-regulation of beta-cell transcription factors would lead to impaired insulin secretion and increased apoptosis in beta-cells (**Figure 1A**). In addition, the glucose-induced repressor of insulin gene transcription *Cebpb* [26] was suggested to be a downstream target of CART. Furthermore, KIR6.2 and SUR1, encoded by *Abcc8* and *Kcnj11* (appeared also among the top regulated insulin genes), were upregulated after CART KD and predicted to be involved in CART action (**Figure 1A**). Finally, CART KD through IKK, NF-kB, PPARγ-and IRS2 was linked to the activation of insulin-induced GLUT4 translocation to the

plasma membrane and reduction in insulin resistance in adipocytes, skeletal muscle and liver (**Figure 1B**). CART KD also resulted in the differential expression of INSR, Erk1/2, IRS, PI3K, PDK1 and AKT and was predicted to inhibit glycogenesis, lipolysis and protein synthesis (**Figure 1B**).

4. DISCUSSION

CART increases insulin secretion in several experimental settings, including human islets and *in vivo* in mice [7, 9]. CART also promotes beta-cell survival during glucotoxic conditions [8]. However, the exact mechanisms behind these effects are yet to be elucidated. In a previous study, we silenced CART in INS-1 (832/13) cells to explore the role of endogenous CART in the beta-cell. This resulted in reduced insulin secretion due to lower ATP levels and reduced exocytosis of docked and primed granules [6]. This was likely related to reduced expression of insulin, exocytotic genes, as well as a network of *Tcf7l2*, *Pdx-1*, *Isl1*, *NeuroD1*, *Mafa*, *Nkx2.2*, and *Nkx6.1* transcription factors [6]. To gain further insight into potential mechanisms explaining the effects of CART on insulin secretion and beta-cell survival, we here further characterized genetic networks affected by CART KD in INS-1 cells using RNAseq.

CART was the most downregulated gene in terms of fold-change, confirming the successful silencing of CART and indicating that the observed differential expression is most likely driven by the silencing of CART. We also compared the overall expression between all the cells using PCA. The most variation, explaining more than 40 percent of the variation in the dataset, was between the CART-silenced cells and the cells treated with scrambled siRNA, indicating that most of the differentially expressed genes are due to CART KD. The fact that CART silencing led to differential expression of more than 25% of all detected genes suggests that CART plays an important role in beta-cells. For comparison, silencing of the transcription factor TCF7L2 in INS-1 (832/13) cells resulted in differential expression of 33% of all detected genes [27]. DAVID pathway analysis suggests that CART KD foremost affects genes involved in processes

related to mitochondria, energy derivation, response to glucose stimulus and cell death. This is largely in line with previous reports on the function of CART in beta- and neuronal cells [6, 8, 24, 25]. In addition, previously uncharacterized roles for CART were identified and included protein transport, nuclear lumen-ER network, macromolecule catabolic processes, and cell junction. Notably, cell communication and synchronization are important for beta-cell function and proper insulin response [28, 29] and we have previously shown that treatment of mouse islets with exogenous CART caused increased synchronization of Ca^{2+} oscillations among beta-cells [7].

One of our main aims was to gain insight into the mechanisms behind the effect of CART on insulin secretion [6, 7, 9, 30]. The most significantly differentially expressed genes related to insulin secretion were *Stx1a*, *Gnas* and *Stxbp1*. Syntaxin 1 (STX1A) is mainly known to be a positive regulator of beta-cell exocytosis [31], but has also been shown to act as a negative regulator of insulin release [32]. Moreover, STX1A together with STXBP are critical for the formation of granular docking sites [33]. Stxbp1 has also been reported to be important for docking [34]. We have previously shown that CART KD in INS-1 (832/13) cells leads to decreased exocytosis of insulin granules in the RRP and affects expression of Stx1a and Stxbp1 [6]. Thus, the present findings further support an important role for CART as a regulator of exocytosis in INS-1 (832/13) cells. *Gnas* is an imrinted gene with important roles in fetal growth and postnatal energy metabolism. *Gnas* generates multiple gene products including transcripts encoding the G protein α-subunit Gsα that couples to many receptors for hormones, neurotransmitters and other extracellular signals to adenylyl cyclase [35]. *Gnas* was downregulated after CART KD in INS-1 (832/13) cells and mice with beta-cell-specific Gsα deficiency display reduced beta-cell mass and develop insulin-deficient diabetes [36]. Genes related to insulin secretion significantly affected by CART KD also include exocytotic genes e.g. synaptotagmins, *Vamp2*, *Snap25*, *Rab3a*; beta-cell transcription factors and genes important for

insulin processing and secretion e.g. *Pdx-1*, *Neurod1*, *Nkx2*.2, *Abcc8, Kcnj11, Pcsk1, Pcsk2* and *Cpe*. Many of these genes have previously been shown to be regulated by CART KD and are likely to mediate the effect of CART KD on insulin granule exocytosis and insulin expression [6].

Abcc8 and *Kcnj11* encoding SUR1 and KIR6.2, forming ATP-sensitive potassium (K_{ATP}) channels in beta-cells, were among the top regulated insulin secretion genes. They were also predicted to be involved in CART action by the pathway analysis. K_{ATP} channel closure is an essential step in stimulus secretion coupling and sulfonylurea drugs, such as tolbutamide and glibenclamide, that close K_{ATP} channels, are potent insulin secretagogues and widely used T2D drugs [37]. The expression of both *Abcc8* and *Kcnj11* was increased after CART KD suggesting that CART KD may result in increased K_{ATP} channel activity and decreased insulin secretion, contributing to the reduced insulin secretion observed after CART KD in INS-1 (832/13) cells. Intriguingly, CART is overexpressed in beta-cells of patients with nesidioblastosis (N. Wierup and J. Rahier, unpublished data), where in many cases *Abcc8* and *Kcnj11* gene mutations have been identified to be causal [38].

CART KD also decreased the expression of *Cebpb* and IPA analysis predicted *Cebpb* to be a mediator of the effect of CART KD on insulin secretion. CEBPB is a transcriptional repressor with regulatory functions in beta-cells. CEBPB expression is increased in clonal beta-cells cultured at high glucose and in islets in T2D models [19, 39, 40]. It is also involved in the repression of insulin transcription under supraphysiological glucose concentrations [26]. CEBPB has also been shown to induce cell cycle arrest and senescence of tumor cells [41] and to promote mammary epithelial cell differentiation [42]. Thus, opposite to what we expected, *Cebpb* expression was decreased after CART silencing in INS-1 (832/13) cells, perhaps as a compensatory mechanism to restore impaired insulin transcription.

Thus, the predicted action of CART in the present study is in agreement with our previously reported functional data [6] (Paper I) and studies in CART KO mice [30] showing that

downregulation or deletion of CART leads to impairment in GSIS and insulin expression. We also confirmed our previous data [6] on reduced expression of beta-cell transcription factors and other key beta-cell genes confirming the reliability of the RNAseq data.

Another main aim was to assess CART-dependent genetic regulation that could explain the mechanisms behind the effect of CART on cell growth and protection against cell death under toxic conditions.

Many of the differentially expressed genes after CART KD are related to cell growth or cell survival. *Nfkb1* was detected as the most significantly regulated apoptosis gene. Attenuation of NF-kB activation in beta-cells in mice results in impaired glucose-stimulated insulin secretion and perturbed expression of genes required for glucose uptake, oxidative metabolism, and insulin exocytosis [43]. *Jun* encodes a part of the AP-1 transcription factor complex that has been implicated in cell differentiation, proliferation and oncogenic transformation [17]. Another top upregulated gene was *RhoB*, a tumor suppressor controlling cell proliferation, migration and invasion. *RhoB* expression is decreased in a number of cancers. Interestingly, *RhoB* has been shown to be important for the induction of beta-cell loss, as *Rhob* deficient mice are more resistant to streptozotocin–induced diabetes [44]. Thus, *Rhob* exerts effects opposite to those of CART in both cancer cells and beta-cells.

The most downregulated gene (except CART) was *Adam 11* [19], a gene implicated in breast cancer, where CART has been described as a marker of poor prognosis and predictor of disease outcome [23]. CART expression has also been shown to be associated with worse survival in small bovel carcinoid tumors [45].

Thus, a number of genes implicated in cell survival and differentiation of tumor cells appear to be regulated by CART. Functional follow-up studies are warranted to assess whether these genes mediate the effects of CART on beta-cell survival and differentiation.

The prediction tool of the IPA software suggested that under high glucose conditions, ROS and beta-cell transcription factors PDX-1, MAFA and CEBPB would mediate the effect of CART KD on insulin secretion and apoptosis in beta-cells. Notably, exogenous CART has previously been shown to protect beta-cells from glucotoxicity-induced cell death and CART KD resulted in increased apoptosis in INS-1 (832/13) cells [6] . In neuronal cells, CART has been shown to act as an antioxidant, protecting mitochondrial DNA, cellular proteins and lipids from the oxidative action of hydrogen peroxide [25]. Thus, ROS may play a role in CART signaling in beta-cells.

We have previously shown that CART affects insulin action in rat adipocytes. CART potentiated isoprenaline-induced lipolysis and the inhibitory effect of insulin on isoprenaline-induced lipolysis [46]. Interestingly therefore CART KD affected the expression of genes related to insulin signaling; *Fasn*, *Crk*, *Ptpn1* being the most significantly differentially expressed genes. Signaling cascades linked to insulin resistance in liver, adipocyte tissue and skeletal muscle were also predicted to be involved in CART signaling. CART KD was predicted to promote insulin-induced GLUT4 translocation to the plasma membrane via modulating the activity of IKK, NF-kB, PPARγ-and IRS2. This is however contrary to our observations in skeletal muscle, where exogenous CART promotes translocation of GLUT4 to the plasma membrane (N. Wierup and K. Stenkula, unpublished observations) and needs further validation.

CART KD was also predicted to inhibit glycogenesis, lipolysis and protein synthesis and to modulate expression of genes involved in insulin signaling. This gains support from previous data showing that exogenous CART phosphorylates CREB, IRS, PKB/FoxO1 and p44/42 MAPK/p90RSK in INS-1 (832/13) cells and rat islets [8].

It should be mentioned that the current analysis of the differentially expressed genes was performed based on p-value without introducing the fold-change cut-off. This approach was selected in order to characterize all the CART KD-activated changes and gene networks. Future

studies using the fold-change cut-off will most likely be useful for identifying the most prominent effects and pathways.

In summary, we have characterized CART-activated genes and functional clusters in INS-1 (832/13) cells. CART was shown to modulate expression of genes with important roles in insulin secretion, insulin signaling, apoptosis and cell cycle regulation. Molecular mechanisms of cancer, PKA signaling, cardiac hypertrophy, insulin receptor signaling, AMPK signaling and T2D diabetes signaling were among the most affected pathways after CART KD. Functional follow-up studies are needed to understand whether the predicted pathways translate into functional pathways.

Figures

Table 1. The top 50 genes displaying differential expression after CART KD in INS-1 (832/13) cells sorted based on fold change (FC). **A**. Downregulated. **B**. Upregulated.

A

gene	FC	p	padj
Cartpt	**5,36**	7,10E-31	8,09E-29
Adam11	4,80	3,16E-31	3,69E-29
Pak3	4,58	1,70E-52	6,44E-50
Ppp1r17	4,43	1,20E-24	8,28E-23
Btbd17	3,91	1,66E-30	1,77E-28
Trim72	3,80	1,45E-17	5,58E-16
Igsf11	3,74	2,60E-31	3,07E-29
Lmbrd2	3,57	1,53E-78	1,68E-75
Hpca	3,56	3,10E-23	1,93E-21
AC142182.1	3,53	1,03E-44	2,49E-42
Scrt1	3,44	1,44E-38	2,47E-36
Ifi27l2b	3,42	1,14E-25	8,36E-24
Gpd1l	3,21	1,36E-75	1,34E-72
Lgi2	3,13	1,09E-42	2,19E-40
Panx2	3,11	2,14E-50	7,32E-48
Adamts8	3,10	3,06E-17	1,13E-15
Pdzd3	3,09	4,51E-16	1,49E-14
Mustn1	3,07	3,06E-17	1,13E-15
Neurod4	3,06	1,96E-21	1,03E-19
Gpr63	3,04	1,50E-16	5,23E-15
Akap2	3,00	8,27E-15	2,44E-13
Rarres2	2,98	9,64E-14	2,49E-12
AABR07038971.1	2,96	2,13E-12	4,65E-11
Nrep	2,93	1,88E-54	8,06E-52
Ifng	2,91	1,94E-28	1,76E-26
Gm2a	2,90	1,69E-53	6,79E-51
Glipr2	2,90	2,81E-26	2,17E-24
Lynx1	2,89	1,14E-30	1,26E-28
Grm4	2,87	3,58E-18	1,43E-16
Trpv1	2,86	6,12E-18	2,43E-16
Rpl3l	2,85	3,05E-74	2,86E-71
Enpp2	2,82	3,78E-46	9,81E-44
Msn	2,80	9,29E-27	7,44E-25
Il22	2,80	3,89E-17	1,43E-15
Baiap3	2,79	1,13E-93	3,18E-90
Tmem178a	2,74	8,31E-14	2,17E-12
St3gal1	2,73	3,09E-65	1,79E-62
Ctnna2	2,73	3,35E-12	7,19E-11
Cx3cl1	2,72	9,70E-28	8,34E-26
Gng8	2,72	8,32E-13	1,89E-11
RX	2,71	2,80E-93	6,90E-90
Mbnl2	2,71	2,21E-71	1,82E-68
Blnk	2,69	1,93E-09	2,82E-08
Cyp4f1	2,67	1,12E-12	2,50E-11
Spock2	2,67	1,29E-52	4,96E-50
Bend6	2,66	2,71E-54	1,14E-51
Gng7	2,65	1,80E-36	2,73E-34
Cops8	2,64	3,46E-98	1,36E-94
Cd40	2,62	8,09E-13	1,84E-11
Nfic	2,60	1,03E-85	1,69E-82

B

gene	FC	p	padj
AABR07068253.1	0,22	8,26E-82	1,02E-78
Jun	0,23	1,51E-43	3,32E-41
Srp14	0,24	4,76E-58	2,34E-55
Sfta2	0,29	3,53E-14	9,67E-13
Apmap	0,31	1,51E-53	6,20E-51
F3	0,33	1,19E-48	3,67E-46
LOC100910404	0,34	3,03E-27	2,51E-25
Ndnf	0,34	2,24E-13	5,49E-12
Ldhb	0,34	4,25E-14	1,15E-12
Rhob	0,35	1,32E-49	4,40E-47
Ffar4	0,36	2,27E-14	6,31E-13
Akap12	0,37	5,29E-70	4,01E-67
P2ry10	0,37	6,47E-10	1,02E-08
Ube2m	0,38	4,12E-158	8,11E-154
Ripply3	0,39	1,61E-44	3,83E-42
AABR07060293.1	0,40	3,57E-53	1,40E-50
Acpp	0,40	1,20E-15	3,83E-14
Il10ra	0,40	7,06E-25	4,98E-23
Tmprss13	0,40	5,25E-24	3,40E-22
Rsad2	0,40	5,03E-14	1,34E-12
Zdhhc9	0,40	2,97E-66	1,83E-63
Insig1	0,40	5,48E-58	2,63E-55
Gpm6a	0,41	1,97E-14	5,53E-13
Potec	0,41	2,66E-08	3,29E-07
Psma1	0,41	3,20E-97	1,05E-93
Rn50_X_0744.6	0,41	2,29E-10	3,85E-09
Dpy19l2	0,42	1,90E-09	2,79E-08
Gstm2	0,42	3,60E-44	8,15E-42
Tle3	0,43	7,34E-31	8,31E-29
Tmem38a	0,43	2,61E-24	1,76E-22
Vom2r12	0,44	3,25E-84	4,92E-81
Tex264	0,44	1,09E-44	2,62E-42
Hsbp1	0,45	2,19E-21	1,14E-19
L1cam	0,45	6,20E-19	2,65E-17
Gad2	0,45	2,58E-15	7,98E-14
Sec23a	0,45	3,58E-36	5,30E-34
Sgpp2	0,45	1,10E-21	5,96E-20
Plagl2	0,45	6,10E-77	6,32E-74
Celf5	0,46	4,75E-28	4,23E-26
Tnfrsf19	0,46	9,99E-07	9,43E-06
Ntm	0,46	1,86E-12	4,09E-11
Nrp2	0,46	5,32E-13	1,24E-11
Map9	0,46	1,54E-45	3,90E-43
Tsx	0,46	3,90E-10	6,35E-09
Fam89a	0,47	1,06E-29	1,06E-27
Styk1	0,47	3,15E-06	2,71E-05
AABR07058699.2	0,47	2,53E-24	1,73E-22
Hcar1	0,47	3,15E-06	2,71E-05
S100a5	0,48	1,09E-06	1,02E-05
AC128848.1	0,48	5,66E-12	1,18E-10

Table 2. The differentially expressed insulin secretion/exocytosis genes after CART KD in INS-1 (832/13) cells sorted based on adjusted p-value (padj). FC=fold change.

gene	FC	p	padj
Stx1a	0,70	2,46E-26	1,92E-24
Gnas	2,49	3,28E-21	1,68E-19
Stxbp1	1,57	7,50E-20	3,47E-18
Pcsk1	0,55	1,51E-18	6,27E-17
Syt4	0,68	2,90E-18	1,18E-16
Neurod1	1,27	5,87E-12	1,21E-10
Camk2a	2,22	9,27E-12	1,88E-10
Camk2b	0,73	2,91E-11	5,52E-10
Syt10	2,38	6,42E-11	1,16E-09
Cacna1d	1,19	7,24E-11	1,29E-09
Nkx6-1	0,68	1,59E-08	2,05E-07
Syt14	1,36	4,47E-08	5,37E-07
Rab3a	0,80	5,72E-08	6,72E-07
Syt7	0,76	8,55E-08	9,72E-07
Nkx2-2	1,34	1,24E-07	1,38E-06
Gip	0,85	1,36E-07	1,50E-06
Adcyap1r1	1,58	5,21E-07	5,16E-06
Kcnj11	0,68	5,69E-07	5,59E-06
Abcc8	0,78	3,01E-06	2,61E-05
Camk2d	1,18	3,60E-06	3,07E-05
Kcnmb2	1,41	7,41E-06	5,93E-05
Plcb1	0,83	9,57E-06	7,50E-05
Syt15	1,46	1,14E-05	8,82E-05
Ryr2	1,36	1,14E-05	8,82E-05
Adcy5	1,34	1,28E-05	9,76E-05
Adcy6	1,23	1,74E-05	1,29E-04
Adcy3	1,28	3,33E-05	2,33E-04
Atf6b	0,89	5,07E-05	3,43E-04
Atp1a4	1,25	6,49E-05	4,27E-04
Atf2	0,84	1,04E-04	6,53E-04
Pclo	1,32	1,76E-04	1,04E-03
Pdx1	1,14	1,81E-04	1,06E-03
Creb5	0,56	4,38E-04	2,34E-03
Prkacb	0,85	5,46E-04	2,83E-03
Isl1	1,23	7,49E-04	3,75E-03
Prkca	0,82	9,53E-04	4,61E-03
Sytl4	1,30	9,83E-04	4,74E-03
Cacna1s	0,84	1,09E-03	5,19E-03
Kcnn1	0,79	1,15E-03	5,43E-03
Rims2	1,14	1,50E-03	6,82E-03
Rims2	1,14	1,50E-03	6,82E-03
Atp1a3	1,24	1,82E-03	8,07E-03
Cpe	1,17	1,85E-03	8,18E-03
Pcsk2	1,25	4,55E-03	1,75E-02
Plcb3	1,14	4,93E-03	1,88E-02
Syt12	1,29	6,28E-03	2,31E-02
Syt12	1,29	6,28E-03	2,31E-02
Mafa	1,50	6,96E-03	2,53E-02
Tcf7l2	1,32	7,42E-03	2,67E-02
Creb3l1	1,31	1,31E-02	4,33E-02
Adcy9	1,18	1,36E-02	4,48E-02
Vamp2	1,11	1,51E-02	4,88E-02

Table 3. The differentially expressed genes related to insulin signaling after CART KD in INS-1 (832/13) cells sorted based on adjusted p-value (padj). FC=fold change.

gene	FC	p	padj
Fasn	0,622	1,19E-43	2,63E-41
Crk	0,710	1,12E-36	1,72E-34
Ptpn1	0,632	9,64E-30	9,74E-28
Rhoq	1,606	9,49E-25	6,58E-23
Pik3r2	1,409	6,09E-23	3,73E-21
Nras	0,704	6,49E-23	3,96E-21
Akt3	0,696	3,44E-19	1,51E-17
Pik3ca	0,717	4,70E-19	2,04E-17
Pygb	0,587	4,27E-14	1,15E-12
G6pc3	0,726	4,70E-14	1,26E-12
Acacb	1,351	6,42E-13	1,47E-11
Gsk3b	0,822	2,90E-11	5,51E-10
Tsc1	0,791	1,26E-10	2,18E-09
Irs1	1,722	7,73E-10	1,20E-08
Cblb	1,367	3,38E-09	4,76E-08
Ppp1ca	0,897	7,49E-09	1,01E-07
Mknk2	0,832	3,63E-08	4,41E-07
Braf	1,298	3,95E-08	4,78E-07
Prkci	0,840	9,12E-08	1,03E-06
Shc2	1,449	1,24E-07	1,37E-06
Pdpk1	0,819	1,24E-07	1,38E-06
Shc3	1,677	1,39E-07	1,52E-06
Mapk9	1,189	1,65E-07	1,79E-06
Fbp1	0,693	1,99E-07	2,13E-06
Ppp1r3c	1,731	2,29E-07	2,42E-06
Eif4e2	0,808	5,58E-07	5,49E-06
Grb2	0,848	1,21E-06	1,12E-05
Elk1	0,838	1,37E-06	1,27E-05
Foxo1	1,227	2,25E-06	1,99E-05
Ptprf	1,199	3,16E-06	2,72E-05
Srebf1	1,195	3,98E-06	3,36E-05
Calm3	0,909	5,16E-06	4,27E-05
Hras	0,849	7,44E-06	5,96E-05
Prkag2	1,271	8,30E-06	6,58E-05
Map2k1	1,235	9,72E-06	7,61E-05
Rheb	1,149	1,29E-05	9,81E-05
Phkg2	1,176	1,63E-05	1,21E-04
Flot2	0,828	3,84E-05	2,66E-04
Exoc7	1,284	4,93E-05	3,34E-04
Prkab1	1,154	6,16E-05	4,08E-04
Mapk3	1,137	9,01E-05	5,73E-04
Gys1	1,287	1,31E-04	8,03E-04
Irs3	1,560	1,92E-04	1,12E-03
G6pc	1,843	1,98E-04	1,15E-03
Eif4e	0,913	2,75E-04	1,54E-03
Rapgef1	0,886	3,19E-04	1,76E-03
Prkab2	0,859	3,50E-04	1,91E-03
Socs3	1,291	3,72E-04	2,02E-03
Bad	1,136	6,17E-04	3,16E-03
Akt1	0,892	7,69E-04	3,83E-03
Phkg1	0,798	9,75E-04	4,71E-03
Ppp1r3d	0,841	1,03E-03	4,92E-03
Prkaa2	0,689	1,03E-03	4,92E-03
Sorbs1	1,168	1,18E-03	5,55E-03
Raf1	0,929	1,34E-03	6,19E-03
Sos2	0,849	1,51E-03	6,85E-03
Prkar2a	0,876	2,87E-03	1,19E-02
Trip10	1,318	4,62E-03	1,78E-02
Inppl1	1,105	5,46E-03	2,05E-02
Pygm	1,409	5,65E-03	2,11E-02
Mapk10	1,124	5,80E-03	2,16E-02
Phka1	1,077	6,84E-03	2,49E-02
Eif4e1b	1,271	1,13E-02	3,83E-02
Mtor	1,207	1,14E-02	3,85E-02
Ppp1cb	1,137	1,31E-02	4,34E-02

Table 4. Genes previously shown to be regulated by CART KD in INS-1 (832/13) cells. FC=fold change.

	FC	pvalue	padj
Slc2a2	0,92	3,62E-01	5,56E-01
Gck	1,00	9,45E-01	9,74E-01
Pcsk1	0,55	0,00E+00	6,27E-17
Pcsk2	1,25	4,55E-03	1,75E-02
Cpe	1,17	1,85E-03	8,18E-03
Ins1	1,31	8,19E-02	1,89E-01
Ins2	1,07	6,37E-01	7,89E-01
Tcf7l2	1,32	7,42E-03	2,67E-02
Mafa	1,50	6,96E-03	2,53E-02
Nkx6-1	0,68	0,00E+00	2,05E-07
Nkx2-2	1,34	0,00E+00	1,38E-06
Isl1	1,23	7,50E-04	3,75E-03
Neurod1	1,27	0,00E+00	1,21E-10
Pdx1	1,14	1,80E-04	1,06E-03
Stx1a	0,70	0,00E+00	1,92E-24
Syt4	0,68	0,00E+00	1,18E-16
Syt7	0,76	0,00E+00	9,72E-07
Syt13	1,14	7,32E-02	1,73E-01
Stxbp1	1,57	0,00E+00	3,47E-18
Vamp2	1,11	1,51E-02	4,88E-02
Snap25	1,01	9,41E-01	9,72E-01

Table 5. The differentially expressed cell cycle genes after CART KD in INS-1 (832/13) cells sorted based on adjusted p-value (padj). FC=fold change.

gene	FC	p	padj
Gadd45g	1,70	2,25E-16	7,63E-15
Crebbp	1,33	2,33E-13	5,69E-12
Anapc1	1,32	2,61E-13	6,32E-12
Ywhab	0,88	9,79E-11	1,72E-09
Cdc14a	1,76	4,15E-10	6,73E-09
Stag2	1,15	1,06E-09	1,63E-08
Anapc10	0,79	1,18E-06	1,10E-05
Mcm4	1,14	3,15E-06	2,71E-05
Bub3	1,14	2,80E-05	2,00E-04
Cdc25c	1,24	3,70E-05	2,57E-04
Cdc20	1,20	4,36E-05	2,98E-04
Anapc11	1,14	1,65E-04	9,85E-04
Ywhag	0,86	4,50E-04	2,39E-03
Orc3	1,16	6,90E-04	3,49E-03
Anapc7	0,87	7,47E-04	3,74E-03
Atm	1,15	8,67E-04	4,26E-03
Mcm2	1,10	1,23E-03	5,75E-03
Anapc2	0,92	1,29E-03	6,02E-03
Cdc26	1,15	1,36E-03	6,29E-03
Atr	0,90	1,51E-03	6,87E-03
Cdc14b	1,12	1,89E-03	8,34E-03
Pkmyt1	0,88	2,68E-03	1,12E-02
Cdc23	0,91	2,91E-03	1,20E-02
Cdc25b	1,12	2,95E-03	1,22E-02
Pttg1	1,14	3,10E-03	1,27E-02
Ccnb1	1,10	9,00E-03	3,15E-02
Gadd45b	0,83	9,43E-03	3,28E-02
Orc2	1,10	1,36E-02	4,47E-02
Ccnh	0,89	1,50E-02	4,84E-02

Table 6. The differentially expressed apoptosis genes after CART KD in INS-1 (832/13) cells sorted based on adjusted p-value (padj). FC=fold change.

gene	FC	p	padj
Nfkb1	1,73	2,16E-50	7,32E-48
Jun	0,23	1,51E-43	3,32E-41
Tuba1a	0,63	7,23E-33	9,19E-31
Nfkbia	1,49	5,38E-25	3,82E-23
Chuk	1,37	5,49E-23	3,38E-21
Sptan1	0,79	9,13E-20	4,19E-18
Dab2ip	1,30	7,55E-19	3,20E-17
Casp3	0,79	1,02E-15	3,25E-14
Cflar	1,66	1,18E-11	2,37E-10
Dffa	0,69	5,44E-10	8,66E-09
Ern1	0,78	7,41E-10	1,16E-08
Ctsz	1,47	1,84E-09	2,71E-08
LOC1003614	0,81	4,04E-09	5,65E-08
Actg1	0,83	5,89E-08	6,90E-07
Htra2	1,26	3,74E-07	3,80E-06
Lmnb1	0,69	5,21E-07	5,16E-06
Diablo	1,21	6,06E-07	5,92E-06
Eif2ak3	0,80	1,78E-05	1,31E-04
Birc5	0,82	1,80E-05	1,33E-04
Eif2s1	0,88	5,39E-05	3,62E-04
Tnfrsf1a	1,27	8,10E-05	5,20E-04
Spta1	0,85	1,01E-04	6,37E-04
Tradd	1,39	2,04E-04	1,18E-03
Casp8	1,80	3,85E-04	2,08E-03
Cycs	0,87	4,43E-04	2,36E-03
Casp9	1,25	5,15E-04	2,69E-03
Itpr1	1,19	1,61E-03	7,23E-03
Bak1	0,88	1,65E-03	7,41E-03
Casp2	0,88	1,99E-03	8,71E-03
Fos	0,85	2,00E-03	8,73E-03
Aifm1	0,89	3,31E-03	1,34E-02
Birc3	1,31	6,26E-03	2,31E-02
Parp2	0,85	6,89E-03	2,50E-02
Ctsl	0,86	9,02E-03	3,16E-02
Daxx	1,09	1,24E-02	4,13E-02

Table 7. Functinal clusters that were upregulated after CART KD in INS-1 (832/13) cells identified by gene ontology (GO) analysis using DAVID.

Annotation Cluster	Enrichment Score		p	padj
1	8.9	nucleotide binding	4.2E-12	6.2E-9
		purine ribonucleotide binding	9.6E-12	7.0E-9
		ribonucleotide binding	1.0E-11	5.0E-9
		purine nucleotide binding	1.0E-11	3.8E-9
		nucleoside binding	1.8E-10	5.4E-8
		purine nucleoside binding	1.5E-9	3.1E-7
		adenyl ribonucleotide binding	1.5E-9	2.8E-7
		ATP binding	1.6E-9	2.6E-7
		adenyl nucleotide binding	2.6E-9	3.8E-7
		nucleotide-binding	2.7E-5	2.1E-3
		atp-binding	7.1E-5	4.9E-3
2	7.78	mitochondrion	8.0E-18	5.2E-15
		organelle membrane	6.9E-11	7.5E-9
		mitochondrial part	9.8E-9	7.0E-7
		organelle envelope	6.7E-8	3.9E-6
		envelope	1.0E-7	5.6E-6
		mitochondrial envelope	5.8E-7	2.7E-5
		mitochondrial membrane	1.2E-6	5.3E-5
		mitochondrion	3.0E-6	4.1E-4
		mitochondrial inner membrane	3.5E-6	1.3E-4
		organelle inner membrane	5.1E-6	1.6E-4
3	7.61	protein transport	1.3E-10	5.4E-7
		protein localization	1.9E-10	4.1E-7
		establishment of protein localization	2.4E-10	3.4E-7
		intracellular transport	8.0E-10	8.5E-7
		protein transport	4.5E-7	8.3E-5
		cellular macromolecule localization	2.9E-6	9.6E-4
		intracellular protein transport	4.5E-6	1.4E-3
		cellular protein localization	4.5E-6	1.3E-3
4	7.33	membrane-enclosed lumen	6.7E-12	1.4E-9
		intracellular organelle lumen	2.3E-11	2.9E-9
		organelle lumen	9.0E-11	7.2E-9
		nuclear lumen	5.1E-7	2.5E-5
		nucleoplasm	3.2E-4	7.8E-3
		nucleoplasm part	5.0E-3	6.6E-2
5	5.96	endomembrane system	4.2E-8	2.7E-6
		nuclear envelope-endoplasmic reticulum network	1.3E-6	5.4E-5
		endoplasmic reticulum part	3.1E-6	1.2E-4
		endoplasmic reticulum membrane	8.3E-6	2.4E-4
6	4.87	energy derivation by oxidation of organic compounds	1.7E-6	6.7E-4
		generation of precursor metabolites and energy	4.8E-6	1.3E-3
		cellular respiration	2.9E-4	1.8E-2

Table 7 (continued). Functinal clusters that were upregulated after CART KD in INS-1 (832/13) cells identified by gene ontology (GO) analysis using DAVID.

Annotation Cluster	Enrichment Score		p	padj
7	4.02	macromolecule catabolic process	2.4E-6	8.4E-4
		protein catabolic process	1.2E-5	2.4E-3
		modification-dependent macromolecule catabolic process	1.4E-5	2.5E-3
		modification-dependent protein catabolic process	1.4E-5	2.5E-3
		cellular macromolecule catabolic process	2.1E-5	3.3E-3
		proteolysis involved in cellular protein catabolic process	2.1E-5	3.2E-3
		cellular protein catabolic process	2.9E-5	3.9E-3
		ubiquitin-dependent protein catabolic process	1.2E-3	4.9E-2
		proteolysis	7.2E-3	1.7E-1
		ubl conjugation pathway	1.0E-1	6.3E-1
8	3.79	programmed cell death	3.0E-5	3.9E-3
		cell death	3.7E-5	4.4E-3
		death	4.2E-5	4.7E-3
		apoptosis	4.3E-5	4.7E-3
		Apoptosis	5.8E-2	5.2E-1
9	3.38	phospholipid metabolic process	7.3E-7	3.4E-4
		organophosphate metabolic process	5.4E-6	1.3E-3
		phospholipid biosynthetic process	1.8E-5	3.0E-3
		glycerophospholipid metabolic process	2.1E-5	3.4E-3
		phosphoinositide metabolic process	2.9E-5	4.0E-3
		glycerolipid metabolic process	3.7E-5	4.4E-3
		phosphoinositide biosynthetic process	7.8E-4	3.7E-2
		GPI anchor metabolic process	8.2E-4	3.8E-2
		protein amino acid lipidation	1.3E-3	5.2E-2
		gpi-anchor biosynthesis	1.4E-3	4.0E-2
		glycerophospholipid biosynthetic process	1.8E-3	6.3E-2
		GPI anchor biosynthetic process	2.4E-3	7.7E-2
		glycerolipid biosynthetic process	3.8E-3	1.1E-1
		lipoprotein biosynthetic process	3.9E-3	1.1E-1
		Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	4.5E-3	6.2E-2
		lipoprotein metabolic process	2.1E-2	3.2E-1
		preassembly of GPI anchor in ER membrane	3.5E-2	4.4E-1
10	3.35	mitochondrial matrix	1.9E-5	5.2E-4
		mitochondrial lumen	1.9E-5	5.2E-4
		transit peptide	7.6E-3	1.5E-1
		transit peptide:Mitochondrion	1.5E-2	1.0E0

Table 8. Functinal clusters that were downregulated after CART KD in INS-1 (832/13) cells identified by gene ontology (GO) analysis using DAVID.

Annotation Cluster	Enrichment Score		p	padj
1	7.08	ion binding	1.5E-10	1.9E-7
		metal ion binding	2.4E-10	1.5E-7
		zinc ion binding	2.6E-10	1.1E-7
		cation binding	1.1E-9	3.4E-7
		transition metal ion binding	3.2E-7	6.7E-5
		metal-binding	1.3E-3	8.0E-2
		zinc	6.6E-3	2.0E-1
2	6.34	neuron projection	6.4E-10	9.3E-8
		cell projection	7.8E-9	9.0E-7
		axon	7.5E-7	4.8E-5
		cell soma	6.8E-5	3.9E-3
		dendrite	8.1E-5	3.6E-3
3	4.83	synapse	3.6E-10	7.0E-8
		cell junction	1.7E-7	1.7E-5
		synapse part	5.7E-7	4.2E-5
		synapse	4.1E-6	7.0E-4
		cell junction	1.0E-4	1.3E-2
		postsynaptic membrane	2.8E-4	8.5E-3
		postsynaptic cell membrane	3.0E-3	1.4E-1
		extracellular ligand-gated ion channel activity	2.0E-1	8.6E-1
4	3.91	enzyme binding	2.3E-5	2.4E-3
		protein kinase binding	2.3E-4	1.2E-2
		kinase binding	3.5E-4	1.4E-2
5	3.89	positive regulation of RNA metabolic process	1.8E-6	2.4E-3
		positive regulation of macromolecule metabolic process	2.1E-6	2.1E-3
		positive regulation of transcription, DNA-dependent	4.4E-6	3.5E-3
		positive regulation of cellular biosynthetic process	5.0E-6	3.4E-3
		positive regulation of macromolecule biosynthetic process	5.5E-6	3.1E-3
		positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	6.1E-6	3.1E-3
		positive regulation of transcription	8.6E-6	3.1E-3
		regulation of RNA metabolic process	1.1E-5	3.7E-3
		positive regulation of transcription from RNA polymerase II promoter	1.1E-5	3.5E-3
		positive regulation of biosynthetic process	1.2E-5	3.4E-3
		positive regulation of nitrogen compound metabolic process	1.3E-5	3.6E-3
		regulation of transcription, DNA-dependent	1.7E-5	4.2E-3
		positive regulation of gene expression	2.3E-5	5.5E-3
		transcription activator activity	2.3E-5	2.2E-3
		regulation of transcription	4.7E-5	9.9E-3
		regulation of transcription from RNA polymerase II promoter	9.6E-5	1.7E-2
		transcription	2.1E-4	3.0E-2
		transcription regulator activity	9.6E-4	3.3E-2
		DNA binding	1.5E-3	4.9E-2
		transcription regulation	1.7E-2	3.0E-1
		Transcription	1.8E-2	3.0E-1
		transcription factor activity	2.5E-2	3.6E-1
		sequence-specific DNA binding	3.8E-2	4.6E-1
		nucleus	6.9E-2	5.2E-1
		dna-binding	3.8E-1	9.0E-1

Table 8 (continued). Functinal clusters that were upregulated after CART KD in INS-1 (832/13) cells identified by gene ontology (GO) analysis using DAVID.

Annotation Cluster	Enrichment Score		p	padj
6	3.75	protein kinase activity	5.5E-8	1.4E-5
		protein amino acid phosphorylation	7.6E-8	1.5E-4
		protein serine/threonine kinase activity	9.5E-7	1.7E-4
		purine ribonucleotide binding	2.6E-6	3.6E-4
		ribonucleotide binding	2.8E-6	3.5E-4
		phosphate metabolic process	6.1E-6	2.7E-3
		phosphorus metabolic process	6.9E-6	2.8E-3
		purine nucleotide binding	9.7E-6	1.1E-3
		phosphorylation	2.4E-5	5.3E-3
		nucleoside binding	3.4E-5	3.1E-3
		nucleotide binding	4.4E-5	3.2E-3
		adenyl ribonucleotide binding	6.9E-5	4.5E-3
		purine nucleoside binding	8.7E-5	5.1E-3
		adenyl nucleotide binding	2.4E-4	1.1E-2
		Serine/threonine protein kinase, active site	2.6E-4	2.1E-1
		ATP binding	2.7E-4	1.2E-2
		kinase	3.8E-4	3.8E-2
		domain:Protein kinase	4.4E-4	4.2E-1
		Serine/threonine protein kinase-related	4.9E-4	2.6E-1
		serine/threonine-protein kinase	8.5E-4	6.9E-2
		Protein kinase, core	9.8E-4	3.6E-1
		Protein kinase, ATP binding site	1.7E-3	4.5E-1
		nucleotide phosphate-binding region:ATP	1.8E-3	4.6E-1
		binding site:ATP	3.6E-3	6.3E-1
		nucleotide-binding	7.8E-3	2.0E-1
		active site:Proton acceptor	1.4E-2	8.8E-1
		Serine/threonine protein kinase	1.8E-2	8.7E-1
		atp-binding	4.0E-2	4.1E-1
		S_TKc	8.1E-2	8.9E-1
		transferase	1.8E-1	7.2E-1
7	3.19	actin filament bundle	1.8E-4	7.3E-3
		actomyosin	3.4E-4	9.3E-3
		stress fiber	4.4E-4	1.0E-2
		actin cytoskeleton	6.5E-3	7.2E-2
8	3.16	membrane fraction	3.9E-4	1.0E-2
		insoluble fraction	5.6E-4	1.2E-2
		cell fraction	1.6E-3	2.5E-2
9	2.98	nucleoplasm	7.9E-5	3.8E-3
		nuclear lumen	8.4E-4	1.5E-2
		intracellular organelle lumen	1.7E-3	2.6E-2
		organelle lumen	1.8E-3	2.6E-2
		nucleoplasm part	2.0E-3	2.8E-2
		membrane-enclosed lumen	3.2E-3	3.9E-2
10	2.74	clathrin-coated vesicle	2.8E-4	8.9E-3
		coated vesicle	6.6E-4	1.3E-2
		membrane-bounded vesicle	2.3E-3	3.0E-2
		cytoplasmic membrane-bounded vesicle	2.4E-3	3.1E-2
		synaptic vesicle	2.1E-2	1.7E-1
11	2.74	response to glucose stimulus	9.3E-4	6.9E-2
		response to monosaccharide stimulus	1.8E-3	9.0E-2
		response to hexose stimulus	1.8E-3	9.0E-2
		response to carbohydrate stimulus	5.4E-3	1.8E-1

Table 9. Canonical pathways affected by CART KD in INS-1 (832/13) cells (p<0.05).

Ingenuity Canonical Pathways	-log(p)	p	Ratio	z-score
Molecular Mechanisms of Cancer	11,2	6,31E-12	0,406	NaN
Protein Kinase A Signaling	10,2	6,31E-11	0,395	1,069
Cardiac Hypertrophy Signaling	9,97	1,07E-10	0,438	2,063
B Cell Receptor Signaling	9,59	2,57E-10	0,459	1,207
Breast Cancer Regulation by Stathmin1	9,51	3,09E-10	0,448	NaN
Germ Cell-Sertoli Cell Junction Signaling	9,23	5,89E-10	0,462	NaN
Role of NFAT in Cardiac Hypertrophy	9,01	9,77E-10	0,448	2,588
GNRH Signaling	8,57	2,69E-09	0,488	-0,126
Ephrin Receptor Signaling	8,26	5,50E-09	0,448	-1,236
Cardiac Î²-adrenergic Signaling	8,05	8,91E-09	0,474	3,539
Insulin Receptor Signaling	8	1,00E-08	0,468	1,364
Rac Signaling	7,78	1,66E-08	0,487	-0,944
Sertoli Cell-Sertoli Cell Junction Signaling	7,72	1,91E-08	0,438	NaN
CD27 Signaling in Lymphocytes	7,7	2,00E-08	0,615	0,186
RANK Signaling in Osteoclasts	7,68	2,09E-08	0,505	1
Signaling by Rho Family GTPases	7,5	3,16E-08	0,405	2,061
Prostate Cancer Signaling	7,47	3,39E-08	0,511	NaN
Axonal Guidance Signaling	7,2	6,31E-08	0,359	NaN
AMPK Signaling	7,1	7,94E-08	0,423	1,778
Acute Myeloid Leukemia Signaling	7,01	9,77E-08	0,505	0
NRF2-mediated Oxidative Stress Response	6,99	1,02E-07	0,42	-1,372
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	6,99	1,02E-07	0,42	2,907
CREB Signaling in Neurons	6,97	1,07E-07	0,424	1,432
Relaxin Signaling	6,72	1,91E-07	0,44	2,16
CXCR4 Signaling	6,71	1,95E-07	0,43	0,378
Pancreatic Adenocarcinoma Signaling	6,71	1,95E-07	0,466	0,617
Type II Diabetes Mellitus Signaling	6,66	2,19E-07	0,457	1,372
14-3-3-mediated Signaling	6,64	2,29E-07	0,454	-1,414
Pyridoxal 5'-phosphate Salvage Pathway	6,56	2,75E-07	0,547	NaN
Prolactin Signaling	6,48	3,31E-07	0,506	0,164
P2Y Purigenic Receptor Signaling Pathway	6,36	4,37E-07	0,447	1,089
Renal Cell Carcinoma Signaling	6,34	4,57E-07	0,506	-0,174
ErbB Signaling	6,28	5,25E-07	0,48	NaN
Colorectal Cancer Metastasis Signaling	6,25	5,62E-07	0,389	2,895

Ingenuity Canonical Pathways	-log(p)	p	Ratio	z-score
Integrin Signaling	5,88	1,32E-06	0,393	-0,57
HGF Signaling	5,88	1,32E-06	0,452	-0,14
fMLP Signaling in Neutrophils	5,86	1,38E-06	0,446	0,429
Glioma Signaling	5,76	1,74E-06	0,455	0,617
Erythropoietin Signaling	5,72	1,91E-06	0,494	NaN
UVA-Induced MAPK Signaling	5,66	2,19E-06	0,461	0,617
Dopamine-DARPP32 Feedback in cAMP Signaling	5,63	2,34E-06	0,414	-0,14
Androgen Signaling	5,62	2,40E-06	0,45	0,655
Neuropathic Pain Signaling In Dorsal Horn Neurons	5,61	2,45E-06	0,447	0,98
NGF Signaling	5,6	2,51E-06	0,444	-0,277
Renin-Angiotensin Signaling	5,6	2,51E-06	0,442	NaN
PPARÎ±/RXRÎ± Activation	5,58	2,63E-06	0,404	0
LPS-stimulated MAPK Signaling	5,48	3,31E-06	0,477	1,093
Endometrial Cancer Signaling	5,46	3,47E-06	0,516	NaN
CD40 Signaling	5,41	3,89E-06	0,487	1,808
Chronic Myeloid Leukemia Signaling	5,37	4,27E-06	0,452	NaN
Huntington's Disease Signaling	5,34	4,57E-06	0,378	-0,13
Thrombin Signaling	5,29	5,13E-06	0,389	1,195
Ceramide Signaling	5,28	5,25E-06	0,462	-1,093
Salvage Pathways of Pyrimidine Ribonucleotides	5,28	5,25E-06	0,462	NaN
Protein Ubiquitination Pathway	5,26	5,50E-06	0,373	NaN
IL-8 Signaling	5,25	5,62E-06	0,391	0,717
Superpathway of Inositol Phosphate Compounds	5,24	5,75E-06	0,381	NaN
Angiopoietin Signaling	5,11	7,76E-06	0,481	-0,408
Non-Small Cell Lung Cancer Signaling	5,11	7,76E-06	0,481	-0,18
Tight Junction Signaling	5,1	7,94E-06	0,401	NaN
CCR3 Signaling in Eosinophils	5,09	8,13E-06	0,423	NaN
ILK Signaling	5,05	8,91E-06	0,388	1,86
Epithelial Adherens Junction Signaling	5,01	9,77E-06	0,411	NaN
SAPK/JNK Signaling	4,96	1,10E-05	0,442	0,603
Superpathway of Cholesterol Biosynthesis	4,96	1,10E-05	0,643	NaN
Cyclins and Cell Cycle Regulation	4,95	1,12E-05	0,474	1,134
Î±-Adrenergic Signaling	4,88	1,32E-05	0,46	1,414
FAK Signaling	4,84	1,45E-05	0,444	NaN

Ingenuity Canonical Pathways	-log(p)	p	Ratio	z-score
mTOR Signaling	4,77	1,70E-05	0,382	0,651
ERK/MAPK Signaling	4,77	1,70E-05	0,382	0,697
Hypoxia Signaling in the Cardiovascular System	4,77	1,70E-05	0,492	-0,302
IL-17 Signaling	4,75	1,78E-05	0,459	NaN
EGF Signaling	4,74	1,82E-05	0,485	0,174
PI3K/AKT Signaling	4,72	1,91E-05	0,419	0,566
IGF-1 Signaling	4,7	2,00E-05	0,434	-0,632
Gap Junction Signaling	4,68	2,09E-05	0,393	NaN
PI3K Signaling in B Lymphocytes	4,61	2,45E-05	0,414	NaN
UVB-Induced MAPK Signaling	4,6	2,51E-05	0,485	1,414
ErbB2-ErbB3 Signaling	4,57	2,69E-05	0,478	NaN
UVC-Induced MAPK Signaling	4,53	2,95E-05	0,548	-0,426
RhoGDI Signaling	4,51	3,09E-05	0,387	-0,816
CDK5 Signaling	4,45	3,55E-05	0,434	-0,762
Endothelin-1 Signaling	4,41	3,89E-05	0,38	1,068
Neurotrophin/TRK Signaling	4,37	4,27E-05	0,461	-0,354
Nucleotide Excision Repair Pathway	4,37	4,27E-05	0,571	NaN
Mouse Embryonic Stem Cell Pluripotency	4,33	4,68E-05	0,425	1,342
IL-1 Signaling	4,33	4,68E-05	0,44	2,121
p70S6K Signaling	4,29	5,13E-05	0,405	0,98
Fc Epsilon RI Signaling	4,24	5,75E-05	0,412	1,732
Estrogen-Dependent Breast Cancer Signaling	4,23	5,89E-05	0,455	NaN
Xenobiotic Metabolism Signaling	4,19	6,46E-05	0,35	NaN
GADD45 Signaling	4,13	7,41E-05	0,684	NaN
G-Protein Coupled Receptor Signaling	4,13	7,41E-05	0,352	NaN
Phosphatidylglycerol Biosynthesis II (Non-plastidic)	4,13	7,41E-05	0,615	NaN
Small Cell Lung Cancer Signaling	4,07	8,51E-05	0,44	NaN
TGF-Î² Signaling	4,07	8,51E-05	0,437	0
PDGF Signaling	4,07	8,51E-05	0,433	-0,801
Glioblastoma Multiforme Signaling	4,03	9,33E-05	0,384	2,177
Role of NFAT in Regulation of the Immune Response	4,02	9,55E-05	0,373	1,905
CDP-diacylglycerol Biosynthesis I	4,01	9,77E-05	0,625	NaN
Cell Cycle: G1/S Checkpoint Regulation	4,01	9,77E-05	0,469	0,6
GABA Receptor Signaling	3,99	1,02E-04	0,463	NaN

Ingenuity Canonical Pathways	-log(p)	p	Ratio	z-score
GM-CSF Signaling	3,96	1,10E-04	0,452	0,898
Semaphorin Signaling in Neurons	3,96	1,10E-04	0,491	NaN
GDNF Family Ligand-Receptor Interactions	3,95	1,12E-04	0,447	1,768
Estrogen Receptor Signaling	3,95	1,12E-04	0,398	NaN
3-phosphoinositide Biosynthesis	3,94	1,15E-04	0,371	NaN
GÎ±q Signaling	3,94	1,15E-04	0,381	1,361
Ovarian Cancer Signaling	3,93	1,17E-04	0,389	NaN
PKCÎ¸ Signaling in T Lymphocytes	3,86	1,38E-04	0,394	1,54
B Cell Activating Factor Signaling	3,83	1,48E-04	0,525	0,471
Amyloid Processing	3,82	1,51E-04	0,49	-0,707
IL-17A Signaling in Airway Cells	3,82	1,51E-04	0,442	2,335
NF-ÎºB Activation by Viruses	3,81	1,55E-04	0,43	NaN
JAK/Stat Signaling	3,81	1,55E-04	0,434	1,667
Ephrin A Signaling	3,74	1,82E-04	0,467	NaN
Virus Entry via Endocytic Pathways	3,73	1,86E-04	0,412	NaN
Docosahexaenoic Acid (DHA) Signaling	3,65	2,24E-04	0,481	NaN
T Cell Receptor Signaling	3,64	2,29E-04	0,404	NaN
Melanoma Signaling	3,63	2,34E-04	0,473	NaN
Sphingosine-1-phosphate Signaling	3,6	2,51E-04	0,393	0,295
Neuregulin Signaling	3,57	2,69E-04	0,42	-1,768
G Beta Gamma Signaling	3,57	2,69E-04	0,42	-0,707
HER-2 Signaling in Breast Cancer	3,57	2,69E-04	0,42	NaN
Myc Mediated Apoptosis Signaling	3,56	2,75E-04	0,443	NaN
Ephrin B Signaling	3,56	2,75E-04	0,438	0,728
Nitric Oxide Signaling in the Cardiovascular System	3,55	2,82E-04	0,398	1,372
Hereditary Breast Cancer Signaling	3,53	2,95E-04	0,38	NaN
Melanocyte Development and Pigmentation Signaling	3,47	3,39E-04	0,411	0,973
HMGB1 Signaling	3,47	3,39E-04	0,383	1,82
IL-3 Signaling	3,44	3,63E-04	0,422	-1,134
IL-6 Signaling	3,43	3,72E-04	0,386	2,143
Leukocyte Extravasation Signaling	3,38	4,17E-04	0,352	1,05
PEDF Signaling	3,32	4,79E-04	0,417	1,567
Role of PKR in Interferon Induction and Antiviral Response	3,31	4,90E-04	0,5	NaN
CNTF Signaling	3,3	5,01E-04	0,444	1,512

Ingenuity Canonical Pathways	-log(p)	p	Ratio	z-score
ERK5 Signaling	3,3	5,01E-04	0,444	-1
Mitotic Roles of Polo-Like Kinase	3,3	5,01E-04	0,439	0,943
RhoA Signaling	3,3	5,01E-04	0,385	-1,372
Role of Tissue Factor in Cancer	3,3	5,01E-04	0,385	NaN
3-phosphoinositide Degradation	3,29	5,13E-04	0,372	NaN
PTEN Signaling	3,28	5,25E-04	0,387	-0,745
VEGF Family Ligand-Receptor Interactions	3,22	6,03E-04	0,409	1,521
FLT3 Signaling in Hematopoietic Progenitor Cells	3,21	6,17E-04	0,412	NaN
Wnt/Î²-catenin Signaling	3,2	6,31E-04	0,361	1,206
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	3,19	6,46E-04	0,418	NaN
Synaptic Long Term Potentiation	3,18	6,61E-04	0,383	0,295
STAT3 Pathway	3,18	6,61E-04	0,425	1,095
Phospholipase C Signaling	3,17	6,76E-04	0,342	0,577
Glioma Invasiveness Signaling	3,17	6,76E-04	0,429	1,461
Cholecystokinin/Gastrin-mediated Signaling	3,17	6,76E-04	0,396	NaN
PAK Signaling	3,17	6,76E-04	0,396	0,973
Lymphotoxin Î² Receptor Signaling	3,17	6,76E-04	0,433	0,962
GÎ±12/13 Signaling	3,16	6,92E-04	0,377	1,313
Telomerase Signaling	3,13	7,41E-04	0,387	1
Reelin Signaling in Neurons	3,12	7,59E-04	0,402	NaN
Factors Promoting Cardiogenesis in Vertebrates	3,12	7,59E-04	0,402	NaN
Apoptosis Signaling	3,11	7,76E-04	0,404	-0,169
Actin Cytoskeleton Signaling	3,09	8,13E-04	0,342	-0,117
Assembly of RNA Polymerase II Complex	3,07	8,51E-04	0,46	NaN
Mitochondrial Dysfunction	3,05	8,91E-04	0,357	NaN
Melatonin Signaling	3,05	8,91E-04	0,423	-0,378
Cholesterol Biosynthesis I	3,04	9,12E-04	0,692	NaN
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	3,04	9,12E-04	0,692	NaN
Cholesterol Biosynthesis III (via Desmosterol)	3,04	9,12E-04	0,692	NaN
Thrombopoietin Signaling	3,04	9,12E-04	0,431	0,756
D-myo-inositol-5-phosphate Metabolism	2,95	1,12E-03	0,362	NaN
Paxillin Signaling	2,94	1,15E-03	0,381	0,973
4-1BB Signaling in T Lymphocytes	2,94	1,15E-03	0,516	1,604
Antiproliferative Role of Somatostatin Receptor 2	2,94	1,15E-03	0,413	1,877

Ingenuity Canonical Pathways	-log(p)	p	Ratio	z-score
Role of MAPK Signaling in the Pathogenesis of Influenza	2,93	1,17E-03	0,417	NaN
ErbB4 Signaling	2,93	1,17E-03	0,417	0,186
Aldosterone Signaling in Epithelial Cells	2,93	1,17E-03	0,355	1,298
Glucocorticoid Receptor Signaling	2,89	1,29E-03	0,328	NaN
cAMP-mediated signaling	2,89	1,29E-03	0,339	3,638
Leptin Signaling in Obesity	2,87	1,35E-03	0,4	1,342
GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	2,87	1,35E-03	0,4	NaN
Regulation of eIF4 and p70S6K Signaling	2,85	1,41E-03	0,357	1,414
Corticotropin Releasing Hormone Signaling	2,83	1,48E-03	0,378	1,298
Adipogenesis pathway	2,83	1,48E-03	0,366	NaN
Role of IL-17A in Arthritis	2,8	1,58E-03	0,418	NaN
HIPPO signaling	2,77	1,70E-03	0,395	1,147
CTLA4 Signaling in Cytotoxic T Lymphocytes	2,75	1,78E-03	0,384	NaN
ATM Signaling	2,74	1,82E-03	0,4	0
Estrogen-mediated S-phase Entry	2,73	1,86E-03	0,542	0
GÎ±s Signaling	2,72	1,91E-03	0,376	2,401
Cell Cycle Regulation by BTG Family Proteins	2,72	1,91E-03	0,486	1,667
Chemokine Signaling	2,69	2,04E-03	0,408	0,186
April Mediated Signaling	2,69	2,04E-03	0,474	0,471
Remodeling of Epithelial Adherens Junctions	2,68	2,09E-03	0,412	-2,887
VEGF Signaling	2,68	2,09E-03	0,379	1,219
Triacylglycerol Biosynthesis	2,66	2,19E-03	0,455	NaN
Tec Kinase Signaling	2,65	2,24E-03	0,347	2,48
FcÎ³RIIB Signaling in B Lymphocytes	2,65	2,24E-03	0,434	1,043
Growth Hormone Signaling	2,63	2,34E-03	0,395	0,18
Cellular Effects of Sildenafil (Viagra)	2,62	2,40E-03	0,362	NaN
CD28 Signaling in T Helper Cells	2,54	2,88E-03	0,359	0,603
Regulation of the Epithelial-Mesenchymal Transition Pathway	2,53	2,95E-03	0,339	NaN
Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	2,5	3,16E-03	0,395	1,8
BMP signaling pathway	2,5	3,16E-03	0,395	NaN
RAR Activation	2,47	3,39E-03	0,337	NaN
D-myo-inositol (1,3,4)-trisphosphate Biosynthesis	2,44	3,63E-03	0,55	NaN
IL-2 Signaling	2,43	3,72E-03	0,406	0,408
Synaptic Long Term Depression	2,43	3,72E-03	0,349	1,82

Ingenuity Canonical Pathways	-log(p)	p	Ratio	z-score
Inhibition of Angiogenesis by TSP1	2,41	3,89E-03	0,471	1,069
TNFR1 Signaling	2,39	4,07E-03	0,429	0,218
GÎ±i Signaling	2,37	4,27E-03	0,358	0,667
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2,34	4,57E-03	0,314	NaN
D-myo-inositol (1,4,5)-trisphosphate Degradation	2,3	5,01E-03	0,556	NaN
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	2,3	5,01E-03	0,351	NaN
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	2,3	5,01E-03	0,351	NaN
eNOS Signaling	2,29	5,13E-03	0,342	2,287
Amyotrophic Lateral Sclerosis Signaling	2,29	5,13E-03	0,36	NaN
Mevalonate Pathway I	2,28	5,25E-03	0,615	NaN
Sperm Motility	2,25	5,62E-03	0,352	1,406
Natural Killer Cell Signaling	2,22	6,03E-03	0,352	NaN
Death Receptor Signaling	2,22	6,03E-03	0,37	-0,686
Induction of Apoptosis by HIV1	2,19	6,46E-03	0,4	NaN
Calcium Signaling	2,15	7,08E-03	0,331	0,667
D-myo-inositol (1,4,5)-Trisphosphate Biosynthesis	2,15	7,08E-03	0,481	NaN
Sonic Hedgehog Signaling	2,13	7,41E-03	0,467	0,577
Dopamine Receptor Signaling	2,1	7,94E-03	0,377	1,807
NF-ÎºB Signaling	2,04	9,12E-03	0,328	0,911
Regulation of Actin-based Motility by Rho	2,03	9,33E-03	0,363	-1,414
Phosphatidylethanolamine Biosynthesis II	2,02	9,55E-03	0,667	NaN
Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism	2,01	9,77E-03	0,48	NaN
HIF1Î± Signaling	2	1,00E-02	0,348	NaN
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	1,99	1,02E-02	0,344	1,886
Mitochondrial L-carnitine Shuttle Pathway	1,94	1,15E-02	0,529	NaN
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	1,94	1,15E-02	0,529	NaN
CCR5 Signaling in Macrophages	1,94	1,15E-02	0,377	NaN
Clathrin-mediated Endocytosis Signaling	1,89	1,29E-02	0,32	NaN
1D-myo-inositol Hexakisphosphate Biosynthesis II (Mammalian)	1,89	1,29E-02	0,5	NaN
FcÎ³ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1,88	1,32E-02	0,355	-0,87

Ingenuity Canonical Pathways	-log(p)	p	Ratio	z-score
Cleavage and Polyadenylation of Pre-mRNA	1,87	1,35E-02	0,583	NaN
TCA Cycle II (Eukaryotic)	1,86	1,38E-02	0,478	NaN
Notch Signaling	1,85	1,41E-02	0,421	-0,707
FGF Signaling	1,85	1,41E-02	0,356	0,707
Antiproliferative Role of TOB in T Cell Signaling	1,85	1,41E-02	0,462	NaN
Wnt/Ca+ pathway	1,85	1,41E-02	0,386	1,091
IL-17A Signaling in Fibroblasts	1,85	1,41E-02	0,429	NaN
G Protein Signaling Mediated by Tubby	1,84	1,45E-02	0,438	NaN
Pyrimidine Ribonucleotides Interconversion	1,84	1,45E-02	0,438	NaN
Diphthamide Biosynthesis	1,82	1,51E-02	1	NaN
NADH Repair	1,82	1,51E-02	1	NaN
Methylglyoxal Degradation I	1,82	1,51E-02	1	NaN
Macropinocytosis Signaling	1,77	1,70E-02	0,358	1,177
iCOS-iCOSL Signaling in T Helper Cells	1,77	1,70E-02	0,336	1,859
Endoplasmic Reticulum Stress Pathway	1,72	1,91E-02	0,476	NaN
Circadian Rhythm Signaling	1,71	1,95E-02	0,424	NaN
IL-22 Signaling	1,71	1,95E-02	0,458	0,905
EIF2 Signaling	1,69	2,04E-02	0,314	0,16
Antioxidant Action of Vitamin C	1,66	2,19E-02	0,34	-1,976
Actin Nucleation by ARP-WASP Complex	1,64	2,29E-02	0,375	-0,688
IL-15 Signaling	1,63	2,34E-02	0,355	NaN
Thyroid Cancer Signaling	1,62	2,40E-02	0,4	NaN
Cdc42 Signaling	1,62	2,40E-02	0,317	-1,151
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	1,61	2,45E-02	0,306	NaN
tRNA Splicing	1,61	2,45E-02	0,405	NaN
RAN Signaling	1,59	2,57E-02	0,5	NaN
Parkinson's Signaling	1,59	2,57E-02	0,5	NaN
Pyrimidine Ribonucleotides De Novo Biosynthesis	1,59	2,57E-02	0,412	NaN
DNA damage-induced 14-3-3Ï*f* Signaling	1,57	2,69E-02	0,474	NaN
nNOS Signaling in Neurons	1,56	2,75E-02	0,383	-0,333
IL-17A Signaling in Gastric Cells	1,56	2,75E-02	0,44	NaN
Role of JAK family kinases in IL-6-type Cytokine Signaling	1,56	2,75E-02	0,44	NaN
Regulation of Cellular Mechanics by Calpain Protease	1,55	2,82E-02	0,368	-0,535
Stearate Biosynthesis I (Animals)	1,48	3,31E-02	0,4	NaN
IL-9 Signaling	1,45	3,55E-02	0,378	1,698

Ingenuity Canonical Pathways	-log(p)	p	Ratio	z-score
Zymosterol Biosynthesis	1,44	0,036307805	0,667	NaN
Urate Biosynthesis/Inosine 5'-phosphate Degradation	1,44	0,036307805	0,5	NaN
Role of CHK Proteins in Cell Cycle Checkpoint Control	1,44	0,036307805	0,364	-0,943
Î³-linolenate Biosynthesis II (Animals)	1,42	0,03801894	0,471	NaN
Human Embryonic Stem Cell Pluripotency	1,39	0,040738028	0,315	NaN
p53 Signaling	1,39	0,040738028	0,324	0,2
Acute Phase Response Signaling	1,36	0,043651583	0,308	0
Assembly of RNA Polymerase I Complex	1,33	0,046773514	0,556	NaN
Folate Transformations I	1,33	0,046773514	0,556	NaN
Role of p14/p19ARF in Tumor Suppression	1,33	0,046773514	0,372	-2,138
Arsenate Detoxification I (Glutaredoxin)	1,31	0,048977882	0,75	NaN
Heme Degradation	1,31	0,048977882	0,75	NaN

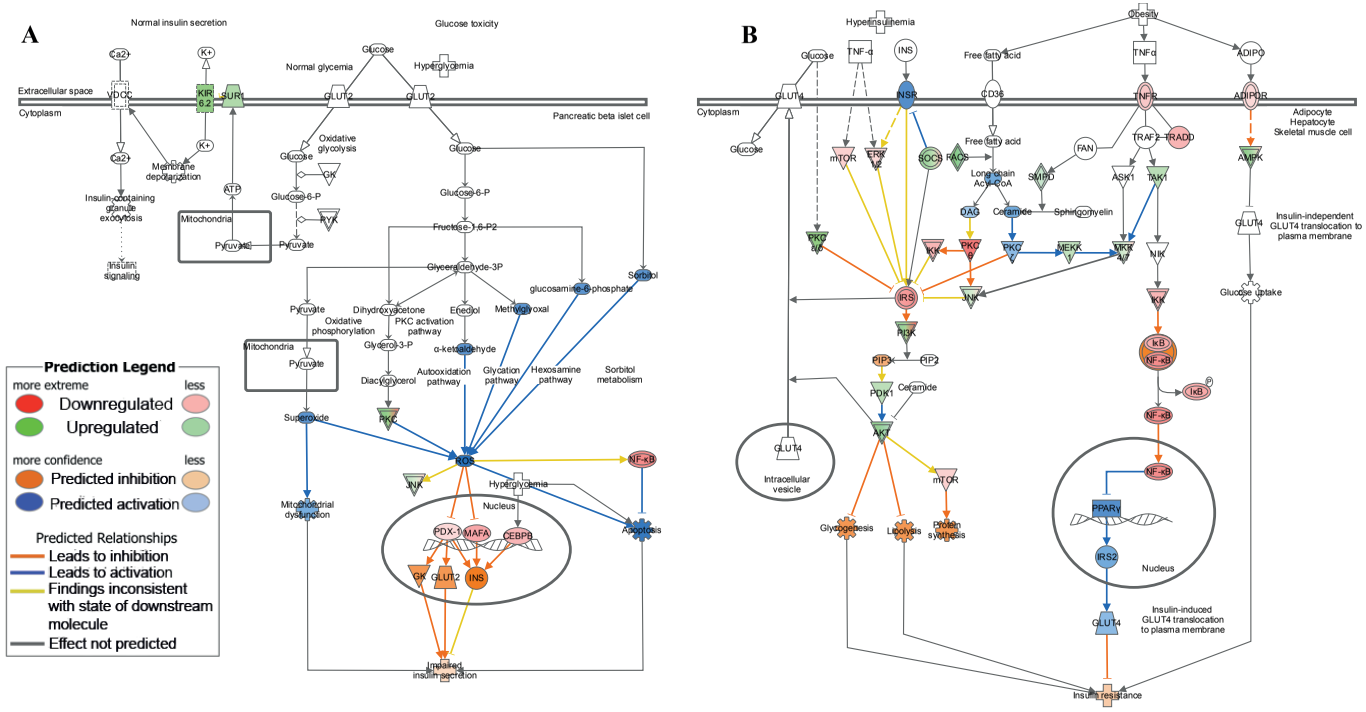

Figure 1. Elements of the canonical T2D signaling pathway in pancreatic beta-cells (**A**) and adipose tissue, skeletal muscle and liver (**B**) that are affected by CART KD in INS-1 (832/13) cells as predicted by pathway analysis. Elements of the pathway displayed in red are downregulated and in green are upregulated; intensity of the color corresponds to the magnitude of the effect on gene expression. Predicted inhibition of the pathway elements is shown in orange and predicted activation is shown in blue; intensity of the color corresponds to the confidence of the predicted effect. Orange arrows indicate predicted downstream inhibition; blue arrows indicate predicted downstream activation.

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Paper III

The Transcriptional Repressor SCRT1 is a novel regulator of beta-cell function

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ABSTRACT

Impaired beta-cell function is a culprit in type 2 diabetes (T2D). T2D islets display decreased glucose-stimulated insulin secretion, and many T2D risk genes have been shown to be important for beta-cell function, beta-cell development, or regulation of beta-cell mass.

Scratch Family Transcriptional Repressor 1 (SCRT1) is a zinc finger transcriptional repressor that binds to E-box motifs. SCRT1 displays a neural-specific expression pattern and may promote neural differentiation. SCRT1 was previously reported as a highly cocaine- and amphetamine regulated transcript (CART)-regulated mRNA in INS-1 (832/13) beta-cells. Here we aimed to characterize islet expression and a potential function of SCRT1 in beta-cells. Our data show that SCRT1 is expressed in INS-1 (832/13) clonal beta-cells and in beta-cells in rodent and human islets. SCRT1 was found to be translocated from the nucleus to the cytosol in diabetic DEX rats and *SCRT1* mRNA expression was reduced by increasing glucose concentrations in INS-1(832/13) cells and human islets. Using siRNA-mediated silencing, we established a role for SCRT1 in regulating insulin expression and secretion. Silencing of *Scrt1* mRNA in INS-1(832/13) cells resulted in impaired glucose-stimulated insulin secretion and decreased insulin expression. This was paralleled by reduced expression of key beta-cell transcription factors *Tcf7l2*, *Pdx-1*, *Isl1*, *Neurod1* and *Mafa*. Furthermore, in human islets expression of *SCRT1* correlated with the expression of insulin, glucagon, beta-cell transcription factors and exocytotic genes. Taken together, our findings suggest that SCRT1 is a novel regulator of insulin secretion.

INTRODUCTION

Type 2 diabetes (T2D) is a pandemic causing spiralling costs for the society and suffering for the patients. Impaired beta-cell function is a hallmark of late stage T2D, but the exact mechanisms behind beta-cell failure are still not fully understood. Therefore, increased knowledge about the regulation of beta-cell function is important and may pave the way for new therapeutic avenues.

Scratch Family Transcriptional Repressor 1 (SCRT1) is a recently identified transcriptional repressor belonging to the SNAIL family of zinc finger transcription factors[1]. Human SCRT1 was isolated from an adult brain cDNA library in 2001[1]. Human SCRT1 shares 92% overall amino acid identity with mouse SCRT1, which is predominantly expressed in newly differentiating, postmitotic neurons and expression persists into postnatal life, suggesting a role for SCRT1 in neuronal differentiation. SCRT1 expression has also been described in the retina, as well as in neuroendocrine cells of the lung in mice. In humans, SCRT1 expression was found in lung tumours with neuroendocrine features [1]. Like other SNAIL family members, SCRT1 binds E-box enhancer motifs "CANNTG", i.e. targets of basic helix-loop-helix (bHLH) transcription factors, leading to transcriptional repression [1]. In 2006, it was reported that SCRT1 is expressed in almost all mouse brain regions, except in motor nuclei and hypothalamic regions, from perinatal stages to adulthood [2]. More recently SCRT1 expression was reported to be induced in newly committed neurons and shown to regulate the onset of neuronal migration via downregulation of E-cadherin [3].

Hitherto SCRT1 expression has not been described in islets and there is no known role of SCRT1 in beta-cell function. In our search for mechanistic basis for the effect of the novel islet hormone cocaine- and amphetamine-regulated transcript (CART) in beta-cell function we identified SCRT1 as a highly CART-regulated mRNA in INS-1 (832/13)

cells. Thus, using RNAseq, we found that the expression of SCRT1 was one of the most significantly reduced transcripts after siRNA-silencing of CART. In the present study, we aimed to assess a potential role of SCRT1 in beta-cell function as well as to study the expression pattern of SCRT1 in human and rodent islets.

MATERIALS AND METHODS

Immunohistochemistry

SCRT1 expression was examined in human and rodent pancreas. The following primary antibodies were used: SCRT1 (Abcam, 1:20; Cambridge, UK; code ab126331), insulin (EuroDiagnostika, Malmö, Sweden; 1:10000; code M9003). Antibodies were diluted in PBS containing 0.25% bovine serum albumin and 0.25% Triton X-100. Slides were incubated with primary antibodies overnight at 4° C, followed by washing in PBS with 0.25% Triton X-100 2 x 10 min. Thereafter, slides were incubated for 1 hour with secondary antibodies conjugated to donkey antirabbit Cy2 (1:400) or donkey antiguineapig Texas Red (1:400) (Jackson ImmunoResearch Laboratories, West Grove, PA). After incubation slides were again washed and then mounted in PBS:glycerol (1:1).

Animal model of type 2 diabetes

Experiments were approved by the Regional Animal Ethics Committee. Female Sprague Dawley rats (225 g) were used. Six rats received dexamethasone (DEX), 2 mg/kg/day intraperitoneally for 10 days [4, 5]. Control rats (n=6) received NaCl 154 mmol/l. The pancreases were processed as described previously [4].

Imaging

Immunofluorescence was examined in an epi-fluorescence microscope (Olympus BX60, Olympus, Tokyo, Japan). Images were taken with a digital camera (Nikon DS-2Mv, Nikon, Tokyo, Japan).

INS-1 (832/13) clonal beta-cell culture

INS-1 (832/13) cells were cultured in RPMI 1640 medium (Sigma) containing 2 g/l D-glucose, supplemented with 10% FBS (Sigma), 10 mM HEPES, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol [6, 7].

siRNA-mediated Silencing and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Silencing in INS-1 (832/131) cells was performed using Lipofectamine RNAiMAX (#13778150, Life Technologies, Carlsbad, California) and 60 nM siRNA targeting rat Scrt1 (s172997, Silencer Select Pre-designed siRNA, Ambion, Life Technologies). The sequences for scrambled siRNA were sense: 5'-GAGACCCUAUCCGUGAUUAtt-3' and antisense: 5'-UAAUCACGGAUAGGGUCUCtt-3' (Silencer Select customer designed siRNA, Ambion, Life Technologies). The transfection complexes were prepared according to the manufacture's protocol. Total RNA was isolated 48 hours after transfection and 1 μg of RNA was reverse-transcribed to cDNA using RevertAid First Strand cDNA synthesis kit (#K1622, Thermo Scientific, Waltham, Massachusetts). qPCR for target genes and two endogenous controls (house-keeping genes TBP and HPRT1 commonly used as reference genes [8]) was performed using 25 ng cDNA and TaqMan Expression PCR Master Mix according to the manufacturer's instructions (Life Technologies). Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. TaqMan

assays used were: *Cart* (Rn01645174_m1), *Tcf7l2* (Hs01009041_g1), *Mafa* (Rn00845206_s1), *Isl-1* (RN00569203_m1), *Pdx-1* (Rn00755591_m1), *NeuroD1* (Rn00824571_s1), *Ins1* (Rn02121433_g1), *Ins2* (Rn01774648_g1), *INS* (Hs00355773_m1), *Hprt* (Rn01527840_m1), *Tbp* (Rn01455646) (Life Technologies).

Acid/ethanol extraction of insulin

INS-1 (832/13) cells were washed twice with PBS 72 hours after siRNA-mediated Scrt1 knock down (KD), lysed in water and sonicated on ice. Thereafter hydrochloric acid/ethanol was added and samples were extracted at -20^{o}C for at least 24 hours. Insulin and proinsulin content was determined using insulin/proinsulin ELISA (Mercodia, Uppsala, Sweden) and normalized to total protein content using the Bio-Rad protein assay (Bio-Rad, Hercules, California).

Glucose-stimulated insulin secretion in INS-1 (832/13) cells

Glucose stimulated insulin secretion (GSIS) was measured 72 hours after SCRT1 KD. Cells were washed twice and incubated in 2.8 mM glucose HEPES-buffered saline solution (HBSS; 114 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.16 mmol/l $MgSO_4$, 20 mmol/l HEPES, 2.5 mmol/l $CaCl_2$, 25.5 mmol/l $NaHCO_3$, 0.2% BSA, pH 7.2), followed by a 1-hour stimulation in the same buffer containing 2.8 mM, 16.7 mM glucose or 16.7 mM glucose with 10 μM IBMX. Insulin concentrations in supernatant were determined using ELISA (Mercodia) and were normalized to total protein content of each well.

***SCRT1* expression in human islets**

Expression of *SCRT1* was examined using RNA sequencing data on human islets from 195 cadaver donors provided by the Nordic Center for Clinical Islet Transplantation in

Uppsala, Sweden and processed as previously described [9]. Correlations between *SCRT1* and beta-cell transcription factors and exocytosis genes in pancreatic islets was analyzed with Pearson's correlation coefficient and corrected for multiple testing (Bonferroni correction).

Statistics

Insulin content data was analyzed using Wilcoxon signed-rank test. Pearson's correlation analysis was performed for correlation studies from human islets. All other experimental data were analyzed using one-way ANOVA, followed by Bonferroni´s post hoc test. GraphPad Prism 7.0 software was used for all statistical analysis. Differences with $p<0.05$ were considered statistically significant.

RESULTS

SCRT1 is expressed in human and rodent pancreatic islets

SCRT1 immunoreactive (IR) cells were evident in both human (**Figure 1 A**) and mouse (**Figure 1 B**) pancreatic islets. Immunostainings with insulin revealed that in both human and mouse pancreas, SCRT1 expression was evident in beta-cells (**Figure 1 A-B**). Using available RNAseq data on transcription levels in sorted human alpha-, beta- and exocrine cells from a previously published study by Bramswig *et al.* [10], we found that *SCRT1* expression was 10-fold higher in beta-cells compared to exocrine cells.

Silencing of SCRT1 in INS-1 (832/13) cells decreases expression of insulin and key beta-cell transcription factors

Since SCRT1 expression was found in human and rodent pancreatic beta-cells, we aimed to investigate the function of SCRT1 in beta-cells using clonal INS-1 (832/13) beta-cells as an *in vitro* model. siRNA-mediated SCRT1 silencing (SCRT1 KD) resulted in a 91.7±3% reduction in *Scrt1 mRNA* (**Figure 2 A**, $p<0.0001$). SCRT1 KD resulted in reduced *Ins1*, *Ins2* and human insulin mRNA levels to 60.2±2.5%, 70±5.2% and 33.5±0.7% respectively (**Figure 2 A**, $p<0.0001$), whereas insulin content was decreased to 83.5±6.1% (**Figure 2 B**, $p<0.05$).

Since SCRT1 silencing decreased insulin expression, we examined expression of beta-cell transcription factors important for insulin transcription. Indeed, expression of *Tcf7l2*, *Isl1*, *Pdx-1*, *Neurod1* and *Mafa* was reduced to 49.3±1.6%, 42.2±1.2%, 57.6±0.6%, 61.7±3.9% and 31.9±3.9% respectively after SCRT1 KD (**Figure 2 C**, $p<0.0001$).

SCRT1-silenced INS-1 (832/13) cells have impaired insulin secretion

Next, we examined glucose stimulated insulin secretion (GSIS) in SCRT1-silenced INS-1 (832/13) cells during static incubations. Increasing the glucose from 2.8mM to 16.7 mM resulted in an approximately 2.8-fold increase in insulin secretion in control cells. SCRT1 KD had no effect on basal insulin secretion (2.8 mM glucose), but led to reduced insulin secretion in cells stimulated with 16.7 mM glucose or IBMX in combination with 16.7 mM glucose (60.4±4.1% and 73.0±2.8% respectively; **Figure 3**).

SCRT1 mRNA is regulated by glucose and SCRT1 is translocated to the cytoplasm in diabetic rats

To assess a potential differential regulation of SCRT1 in T2D we assessed SCRT1 expression in diabetic DEX rats [4, 5]. In vehicle-treated control rats SCRT1 IR was evident in the majority of beta-cell nuclei (**Figure 4**). In hyperglycemic DEX rats, on the other hand, SCRT1 was mainly localized to the cytoplasm (**Figure 4**). These data suggest that SCRT1 expression may be regulated by glucose. Therefore, we assessed *Scrt1* expression in INS-1(832/13) cells cultured at increasing concentrations of glucose. This revealed that *Scrt1* mRNA was reduced by 11.1, 16.7 and 25 mM glucose compared with cells cultured at 5.5 mM glucose (to 60.09±6.7%, $p<0.01$; 47.9±4.5%, $p<0.001$ and 42.8±3.5%, $p<0.001$ respectively; **Figure 5 A**). Finally, we assessed *SCRT1* expression in human islets cultured in media containing high or low glucose concentrations. *SCRT1* mRNA levels were found to be decreased to 44.6±10.8% ($p<0.05$) after culture in high glucose also in human islets (**Figure 5 B**).

SCRT1 expression correlates with insulin, glucagon, and genes important for beta-cell function in human islets

As SCRT1 was found to regulate insulin expression and secretion, as well as expression of important beta-cell transcription factors in INS-1 (832/13) cells, we next wanted to assess whether SCRT1 plays a role in human islet function. To this end, we performed correlation analysis to assess whether *SCRT1* expression correlates with genes important for beta-cell function in available RNAseq data from 195 human islets donors[9]. *SCRT1* expression correlated positively with insulin (**Figure 6 A**), glucagon (**Figure 6 B**), and the transcription factors *MAFA*, *PDX-1*, *ISL1* and *NEUROD1* (**Figure 6 D-G**), but negatively with *TCF7L2* (**Figure 6 C**). In addition, *SCRT1* expression correlated with expression of genes involved in regulation of insulin expression and processing, as well as glucose sensing (**Table 1**). Moreover, *SCRT1* mRNA expression correlated with expression of genes important for beta-cell exocytosis, including syntaxins and synaptotagmins (**Table 1**). Thus, although we have no causal evidence, these observations suggest that SCRT1 may be of importance also in human beta-cells. Finally, there was no significant difference in *SCRT1* transcript levels between control and T2D donors, but *SCRT1* expression correlated negatively with HbA1c ($p=0.037$, $R^2=0.024$).

DISCUSSION

Here we show that SCRT1 is a novel constituent of human and rodent islets that may play an important role in beta-cell function. Thus, SCRT1 expression was evident in beta-cells of humans, rats and mice. SCRT1 was found to be translocated from the nucleus to the cytosol in diabetic rats and *SCRT1* mRNA expression was reduced by increasing glucose concentrations in INS-1 (832/13) cells and human islets. Furthermore, silencing of SCRT1 resulted in reduced insulin secretion, as well as reduced insulin gene and protein expression in parallel with reduced expression of key beta-cell transcription factors.

In INS-1 (832/13) cells, silencing of SCRT1 robustly decreased insulin gene expression and insulin content. Furthermore, glucose-stimulated insulin secretion was robustly decreased after SCRT1 silencing. Taken together our data suggests that both impaired insulin release and insulin production explain the observed reduction in insulin secretion. Interestingly, SCRT1 and also its paralog and another SNAIL family member SCRT2 were shown to downregulate E-cadherin. In the beta-cell E-cadherin is an important mediator of adherent junction and E-cadherin-mediated cell adhesion was shown to be essential for beta-cell viability and insulin secretion [11-14]. Our preliminary data suggest that *Cdh1* expression is decreased after SCRT1 KD (unpublished observations). This is opposite to the previously reported negative regulation of *Cdh1* expression by SCRT1 [3]. Nevertheless, the observed reduction in *Cdh1* expression is compatible with reduced insulin secretion and may partially explain the reduced GSIS after SCRT1 KD.

We also showed that SCRT1 KD reduced expression of *Tcf7l2*, *Isl1*, *Pdx-1*, *Neurod1* and *Mafa*, all important transcription factors regulating insulin gene expression. This could possibly explain the effect of SCRT1 KD on insulin transcription. The exact

mechanisms behind the effect of SCRT1 is not known, but the ability of SCRT1 to interact with E-box sequences of DNA promoters may suggest binding of SCRT1 directly to the E-box in the insulin promotor. Alternatively, SCRT1 may also interact with histone deacetylases and modify chromatin structure, as was shown for Snail-regulated E-cadherin expression [15]. Additionally, SCRT1 may regulate insulin transcription by binding to the E-motif- containing promotors of beta-cell transcription factors e.g. PDX-1 [16]. ChIP experiments are warranted to identify transcriptional targets of SCRT1 in beta-cells. Interestingly, therefore our preliminary data suggest binding of SCRT1 to the E-boxes in the *Ins1* promoter (S. Chriett, unpublished observations) and thus direct regulation insulin transcription. We also found SCRT1 expression to correlate with *INS*, *GCG* genes, beta-cell transcription factors and exocytotic genes in human islets. This suggests, but does not prove, that SCRT1 may regulate these genes also in human islets.

SCRT1 was identified as a highly downregulated mRNA in RNA-seq analysis of CART-silenced INS-1 (832/13) cells [17]. Silencing of either SCRT1 or CART in INS-1 (832/13) cells impairs insulin expression and secretion and reduced the expression of the transcription factors *Tcf7l2*, *Isl1*, *Pdx-1*, *Neurod1* and *Mafa*. Furthermore, in human islets there was a large degree of overlap in the correlations with key beta-cell genes for CART and SCRT1. Based on these observations it is tempting to speculate that SCRT1 is a mediator of the effects of CART on insulin in INS-1 (832/13) cells. When comparing the effect of KD of CART or SCRT1 some differences were apparent. Thus, silencing of CART resulted in a robust decrease in insulin transcription and insulin content, with a modest effect on insulin secretion [17], whereas SCRT1 silencing had a robust effect on insulin secretion, and transcription, but only a modest effect on insulin

content was seen. This indicates differences in the mode of action and that SCRT1 is likely not the only mediator of the CART effects.

Furthermore, we observed that SCRT1 was translocated from the nucleus to the cytosol in diabetic DEX rats. This is reminiscent of the pattern of the beta-cell transcription factor PDX-1, which is translocated from nucleus to the cytosol in rodent models of metabolic stress [18-22]. On the contrary glucose and stimulators of insulin secretion and beta-cell survival increase nuclear PDX-1 [23-25]. The observed translocation of SCRT1 suggest impairment in SCRT1 shuttling from the cytosol to the nucleus in the diabetic state that may lead to decreased SCRT1 transcriptional activity. Based on our findings suggesting that SCRT1 is a positive regulator of several important beta-cell genes, the translocation is compatible with impaired beta-cell function. In addition, *Scrt1* mRNA was negatively regulated by glucose in INS-1 (832/13) cells and human islets, and *SCRT1* expression correlated negatively with HbA1c in human islets. This suggests that reduced SCRT1 expression may play a role in mediating the detrimental effects of glucotoxicity and oxidative stress on insulin expression and secretion as shown for e.g. MAFA and PDX-1 [26, 27].

In summary, SCRT1 is a novel glucose-regulated constituent of beta-cells in humans and rodents. Our data suggest that SCRT1 is a stimulator of insulin secretion and transcription. In view of these data, the observed differential regulation of SCRT1 in a rodent model of T2D is intriguing and suggests that SCRT1 may play a role in T2D pathogenesis.

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FIGURES

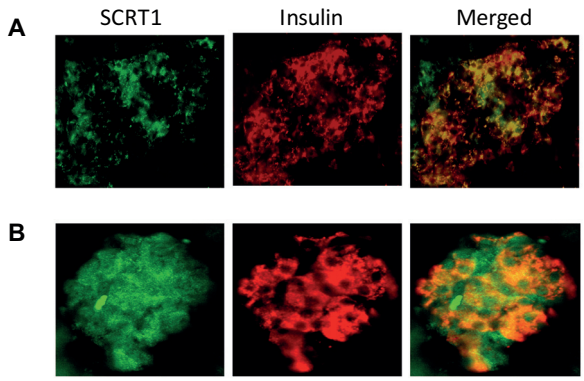


Figure 1. Human and mouse pancreatic sections, double immunostained for SCRT1 (green) and insulin (red). SCRT1 is expressed in human (**A**) and mouse (**B**) beta-cells.

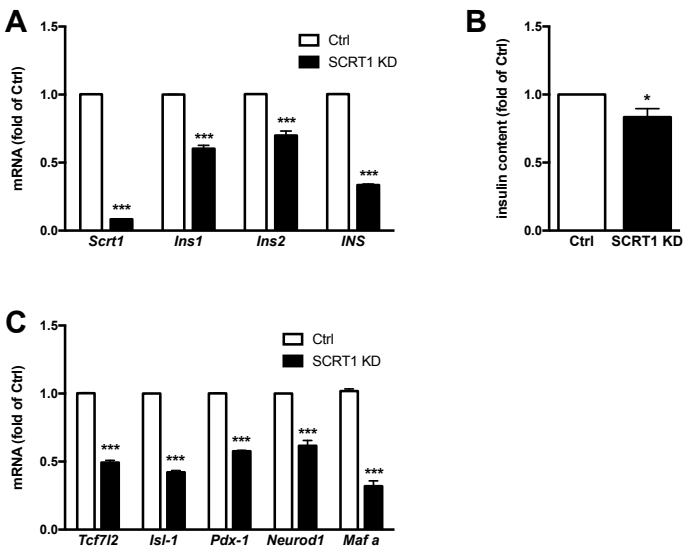


Figure 2. SCRT1 KD reduces insulin and transcription factor expression in INS-1 (832/13) cells

Decreased *Ins1, Ins2* and *INS* gene expression after SCRT1 KD compared with scrambled siRNA (Ctrl) (**A**). Reduced insulin content after SCRT1 KD (**B**). SCRT1 KD in INS-1 (832/13) cells reduces the expression of beta-cell transcription factors (**C**). Data presented as mean±SEM. *, $p<0.05$; ***, $p<0.001$.

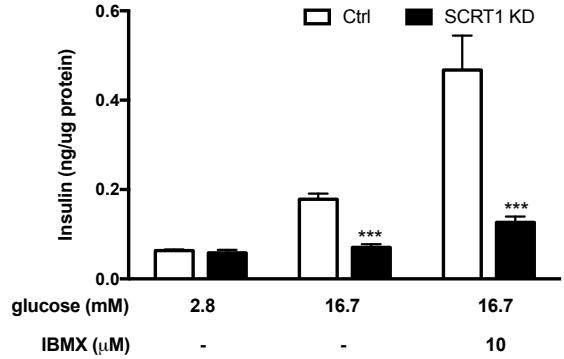


Figure 3. Insulin secretion is reduced in INS-1 (832/13) cells after SCRT1 KD SCRT1 KD reduces insulin secretion stimulated by 16.7 mM glucose and by 16.7 mM glucose combined with 3-isobutyl-1-methylxanthine (IBMX) during 1h static incubations. Data presented as mean±SEM. ***, $p<0.001$.

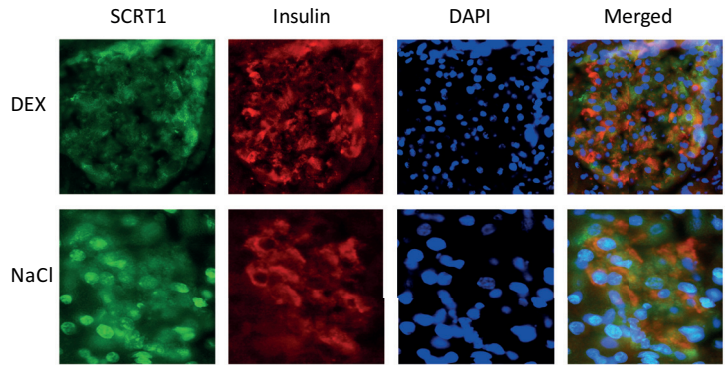


Figure 4. Double immunostaining for SCRT1 (green) and insulin (red), DAPI (blue) in rat pancreatic sections. SCRT1 is translocated to the cytoplasm in the diabetic DEX-treated rats (upper panel) compared with saline-treated control rats (lower panel).

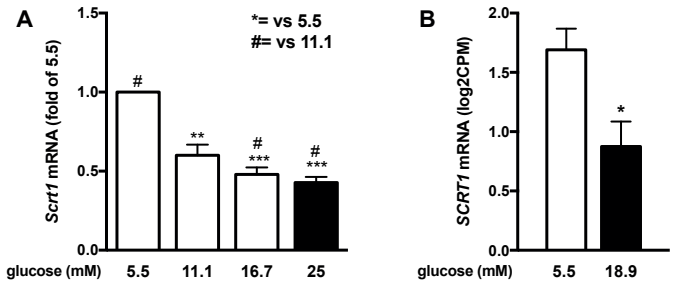
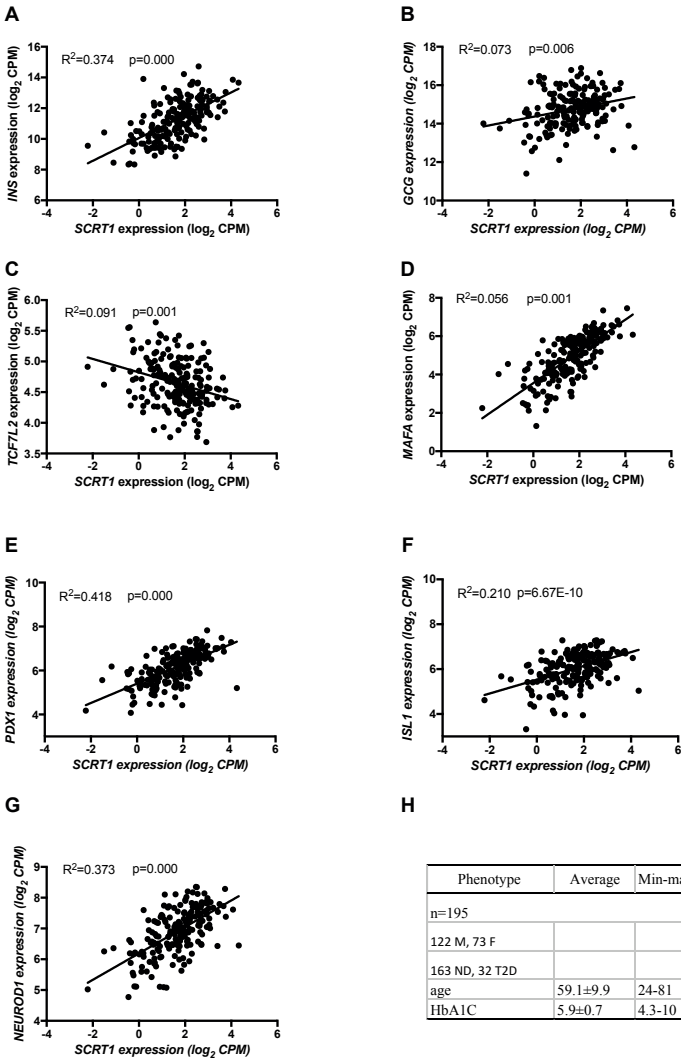


Figure 5. *SCRT1* expression is regulated by glucose in INS-1 (832/13) cells and in human islets

Scrt1 mRNA in INS-1 (832/13 cells) is reduced after 24-h culture in 11.1, 16.7 and 25 mM glucose compared with cells cultured at 5.5 mM glucose (**A**). *SCRT1* mRNA levels are decreased after culture in 18.9 mM glucose in human islets (**B**). Data presented as mean±SEM. *, $p<0.05$; #, $p<0.05$.



H

Phenotype	Average	Min-max
n=195		
122 M, 73 F		
163 ND, 32 T2D		
age	59.1±9.9	24-81
HbA1C	5.9±0.7	4.3-10

Figure 6. *SCRT1* expression correlates with important beta-cell genes in human islets

SCRT1 expression correlated positively with *INS* (**A**), *GCG* (**B**), and the transcription factors *MAFA*, *PDX1*, *ISL1* and *NEUROD1* (**D-G**), but negatively with *TCF7L2* (**C**) in RNAseq data from 195 human islet donors. Islet donor characteristics (**F**). T2D: type 2 diabetic donors; ND: non-diabetic donors; M: male; F: female. P-value was adjusted for multiple testing.

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Table 1. *SCRT1* expression correlates with important beta-cell genes in human islets in RNAseq data from 195 human islet donors.

gene	Correlation coefficient, r	nominal p-value	adjusted p-value	Significant	
				Nominal	Bonferroni corrected
INS	0,61171637610504800000	0,00E+00	0,00E+00	YES	YES
GCG	0,27042207135525500000	1,32E-04	5,66E-03	YES	YES
TCF7L2	-0,30290594997542300000	1,68E-05	7,21E-04	YES	YES
MAFA	0,75223245923285800000	0,00E+00	0,00E+00	YES	YES
MAFB	0,63987836869840400000	0,00E+00	0,00E+00	YES	YES
PDX1	0,64651155780333800000	0,00E+00	0,00E+00	YES	YES
NKX6-1	0,63807549688688400000	0,00E+00	0,00E+00	YES	YES
NKX2-2	0,65907956523921000000	0,00E+00	0,00E+00	YES	YES
ISL1	0,45865351369767100000	1,55E-11	6,67E-10	YES	YES
NEUROD1	0,61102945811802600000	0,00E+00	0,00E+00	YES	YES
GCK	0,59629857488426700000	0,00E+00	0,00E+00	YES	YES
PCSK1	0,30975371093719300000	1,05E-05	4,52E-04	YES	YES
PCSK2	0,52684337934012700000	2,55E-15	1,10E-13	YES	YES
SLC2A1	0,13341505643962900000	6,30E-02	2,71E+00		
CPE	0,28258666300342400000	6,26E-05	2,69E-03	YES	YES
RAB3A	0,74312810464892300000	0,00E+00	0,00E+00	YES	YES
RIMS1	-0,22753176806150300000	1,38E-03	5,93E-02	YES	
RIMS2	0,37416539014208000000	7,11E-08	3,06E-06	YES	YES
SNAP25	0,31647605798905200000	6,56E-06	2,82E-04	YES	YES
STX1A	0,57368138788642900000	0,00E+00	0,00E+00	YES	YES
STXBP1	0,66107676506956800000	0,00E+00	0,00E+00	YES	YES
STXBP2	0,06219694763002030000	3,88E-01	1,67E+01		
STXBP3	-0,32144135499038900000	4,61E-06	1,98E-04	YES	YES
SYT1	0,32861600911042600000	2,73E-06	1,17E-04	YES	YES
SYT10	0,05229318022450990000	4,68E-01	2,01E+01		
SYT11	0,35047958259272100000	5,09E-07	2,19E-05	YES	YES
SYT12	0,02794119324123840000	6,98E-01	3,00E+01		
SYT13	0,45690278262524000000	1,89E-11	8,15E-10	YES	YES
SYT14	0,38898427499130300000	1,91E-08	8,22E-07	YES	YES
SYT15	-0,11502613961408700000	1,09E-01	4,70E+00		
SYT16	0,25104468413515200000	4,00E-04	1,72E-02	YES	YES
SYT17	0,61988400577715300000	0,00E+00	0,00E+00	YES	YES
SYT2	0,03640647887749210000	6,13E-01	2,64E+01		
SYT3	0,47054067599410500000	3,89E-12	1,67E-10	YES	YES
SYT4	0,29563763514274600000	2,72E-05	1,17E-03	YES	YES
SYT5	0,57246343635445800000	0,00E+00	0,00E+00	YES	YES
SYT6	-0,28806437486998200000	4,43E-05	1,91E-03	YES	YES
SYT7	0,68545205742487800000	0,00E+00	0,00E+00	YES	YES
SYT8	0,04959393576460860000	4,91E-01	2,11E+01		
SYT9	0,25067843967808700000	4,08E-04	1,76E-02	YES	YES
SYTL4	-0,15813648431835200000	2,72E-02	1,17E+00	YES	
UNC13B	-0,02650857445099300000	7,13E-01	3,07E+01		
VAMP2	0,30124957656741200000	1,87E-05	8,05E-04	YES	YES

Paper IV

Intestinal CART is a regulator of GIP and GLP-1 secretion and expression

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Abstract

Impaired incretin effect is a culprit in Type 2 Diabetes. Cocaine- and amphetamine-regulated transcript (CART) is a regulatory peptide controlling pancreatic islet hormone secretion and beta-cell survival. Here we studied the potential expression of CART in enteroendocrine cells and examined the role of CART as a regulator of incretin secretion and expression.

CART expression was found in glucose-dependent insulinotropic polypeptide (GIP)-producing K-cells and glucagon-like peptide-1 (GLP-1)-producing L-cells in human duodenum and jejunum and CART levels were increased 60 min after a meal in humans. CART expression was increased by fatty acids and GIP, but unaffected by glucose in GLUTag and STC-1 cells. Exogenous CART had no effect on GIP and GLP-1 expression and secretion in GLUTag or STC-1 cells, but siRNA-mediated silencing of CART reduced GLP-1 expression and secretion. Furthermore, acute administration of CART increased GIP and GLP-1 secretion during an oral glucose tolerance test in mice. We conclude that CART is a novel constituent of human K- and L-cells with stimulatory actions on incretin secretion and that interfering with the CART system may be a therapeutic avenue for T2D.

Keywords: CART, cocaine- and amphetamine-regulated transcript, GIP, GLP-1, enteroendocrine cells, incretin hormones

Introduction

Reduced incretin secretion and action are hallmarks of Type 2 Diabetes (T2D) [1, 2]. It is well established that the insulinotropic effect of glucose-dependent insulinotropic polypeptide (GIP) is reduced in T2D subjects, while obesity is associated with increased GIP levels. Glucagon-like peptide-1 (GLP-1) plasma levels are lower in T2D patients, but its insulinotropic effect is retained in T2D [1, 3-5]. This is the basis for the success of GLP-1 receptor agonists and dipeptidyl peptidase-4 (DPP4) inhibitors in the treatment of T2D patients [2, 3].

Cocaine- and amphetamine-regulated transcript (CART) is a brain-gut peptide with a wide range of biological effects as a neurotransmitter and as a hormone [6, 7]. CART expression has been demonstrated in the central, peripheral and enteric nervous systems [7-9], in adipose tissue [10], as well as in endocrine cells in the pancreatic islets [11, 12], the thyroid [13] and the adrenal medulla [13, 14].

In appetite-regulating nuclei of the hypothalamus CART acts as an inhibitor of food intake [6, 8], and *CART*-/- mice develop obesity [15, 16]. *CART*-/- mice also exhibit impaired glucose tolerance due to insufficient insulin release, prior to the onset of obesity [15]. Furthermore, although the mechanisms are not completely understood, CART increases insulin secretion from isolated human and mouse islets, as well as *in vivo* in mice [17]. In addition, CART further augments GLP-1-enhanced glucose-stimulated insulin secretion [17, 18]. Similar to the incretin hormones, the effect of CART on insulin secretion is glucose-dependent [17]. Furthermore, alike GLP-1, CART decreases glucagon secretion in mouse and human islets and *in vivo* in mice [17] and protects beta-cells from glucotoxicity-induced cell death *in vitro* in rats [19].

In the gastrointestinal (GI) tract CART expression has been described in the enteric nervous system in rats [7], pigs [13], sheep [20], guinea pigs [21] and humans [22]. Less is known about CART expression in enteroendocrine cells. In sheep, a small population of CART immunoreactive (IR) endocrine cells was detected in the

abomasum part of the stomach [20]. In rats, CART-expressing endocrine cells were confined to the antrum and duodenum. The majority of these cells were identical to gastrin-producing G-cells, but a a duodenal subpopulation remains unidentified [7]. Although CART is produced by human neuroendocrine GI tumors [23] there is to this date no information on CART expression in enteroendocrine cells in the normal human GI-tract.

Here we investigated CART expression in K- and L-cells in the human duodenum and jejunum. Silencing of CART expression and exogenous CART peptide treatment in GLUTag and STC-1 cells were used in order to elucidate the role of CART in the regulation of incretin synthesis and secretion. In addition, CART was administered during an OGTT in mice to assess potential effects of CART on glucose-stimulated incretin secretion.

Materials and methods

Human specimens

Human specimens from duodenum and jejunum were collected during surgery. The study was approved by the Regional Ethical Review Board. Specimens were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.2) before being embedded in paraffin. 6-μm sections were cut and mounted on coated slides, deparaffinized and hydrated prior to immunohistochemistry. Normal histology of the specimens was verified with hematoxylin staining.

Immunohistochemistry

The following previously characterized primary antibodies were used: goat anti-CART (1:200, code sc-18068, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-CART (1:1000, code 12/D, kindly provided by Prof. M. J. Kuhar, Emory University, Atlanta, GA) [23], rabbit anti-GIP (1:1200, code 11/19/77, a kind gift from Prof. T. O'Dorisio,

University of Ohio, Columbus, OH) [24], rabbit anti-proglucagon (1:5000, code 7811, EuroDiagnostica, Malmö, Sweden) [24], mouse anti-chromogranin A (1:160, code LK2H10, Boehringer, Mannheim, Germany) [25]. Specificity of the CART antibody was verified with lack of immunoreaction after preabsorption with CART 55-102 peptide (10-100 μg/ml in working dilution), as well as in *CART-/-* mice [23]. Immunohistochemistry was performed as previously detailed [23, 24].

Imaging

Immunofluorescence was examined in an epi-fluorescence microscope (Olympus BX60, Tokyo, Japan), as well as in a laser scanning confocal microscope (Zeiss 510 Meta LSM and a ×63 oil immersion objective (NA=1.25)) and analysis was performed using the ZEN 2009 software (Zeiss, Oberkochen, Germany). Images were taken with a digital camera (Nikon DS-2Mv, Tokyo, Japan). All cells immunoreactive for CART/GIP, CART/GLP-1 or CART/GIP/GLP-1 were analyzed in at least two sections from each specimen (n=9 for duodenum and n=10 for jejunum). For each double-staining 30-600 CART IR cells were counted per subject. The identity of the specimen was unknown to the observer.

Oral glucose tolerance test (OGTT) and mixed-meal test (MMT) in humans

Eight healthy volunteers were subjected to OGTT (6 females and 2 males; age 26-55 years, fasting glucose 4.7-5.5 mM, fasting insulin 2-8 mU/L, BMI 20-27 kg/m^2) and nine volunteers were subjected to MMT (7 females and 2 males; age 26-55 years, fasting glucose 4.6-6.1 mM, fasting insulin 3-8 mU/L, BMI 20-27 kg/m^2). The studies were approved by the Ethical Committee of the Hospital District of Southwestern Finland, and informed consent was obtained from all volunteers prior to participation in the study. The mixed-meal solution (Nutridrink, Nutricia Advanced Medical Nutrition, Amsterdam, Netherlands; 250 kcal, 40 g carbohydrates, 6 g fat, 9 g protein)

was administered orally during a 10-min period after an overnight fasting. Blood samples were collected at 0, 15, 30, 45, 60 and 90 min. For the OGTT, the volunteers had fasted overnight, and a 75-gram oral glucose load was given during 10 minutes. Blood samples were collected at 0, 15, 30, 60, 90 and 120 min. CART plasma levels were determined using a CART EIA (RayBiotech, Norcross, GA) according to the manufacturer's instructions [26].

Cell culture

GLUTag cells (kindly provided by Prof. D. J. Drucker, Mount Sinai Hospital, Toronto, Canada), were used for studies of GLP-1 expression and secretion [27]. GLUTag cells were cultured in DMEM, 1 g/l glucose supplemented with 10% FBS, 2 mM glutamine (Sigma Aldrich, St. Louis, MO). The polyhormonal secretin tumor cell line STC-1 [28] (a kind gift from Prof. J.Y. Scoazec, Edouard Herriot Hospital, Lyon, France) was used for studies of GIP expression and were cultured in DMEM, 4.5 g/l glucose, supplemented with 5% FBS (Sigma Aldrich).

siRNA-mediated CART silencing

CART silencing in GLUTag and STC-1 cells was performed using Lipofectamine RNAiMAX (Life Technologies, Waltham, MA) and 60 nM siRNA targeting mouse CART (#4390815 Silencer Select Pre-designed siRNA, Ambion, Life Technologies, Waltham, MA and J-090320-11-0002, ON-TARGETplus Rat Cartpt siRNA, Dharmacon, Lafayette, CO). Scrambled siRNA was used as negative control (the sequences for scrambled siRNA were sense: 5'-GAGACCCUAUCCGUGAUUAtt-3' and antisense: 5'-UAAUCACGGAUAGGGUCUCtt-3', Silencer Select customer designed siRNA, Ambion, Life Technologies). The transfection complexes were prepared according to the manufacturer's protocol. Total RNA was isolated 48 h after transfection. For protein analysis cells were lysed 72 h after transfection using Lysis-

M reagent supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentration was determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). GLP-1 concentrations were determined using an active GLP-1 ELISA (EMD Millipore Corporation, Billerica, MA) and normalized to total protein content. GIP concentrations were determined using a total GIP ELISA (EMD Millipore Corporation) and normalized to total protein content.

Quantitative real-time polymerase chain reaction (qPCR)

GLUTag and STC-1 cells were seeded in 24-well plates and cultured for 24 h. Thereafter medium was replaced with new medium with or without CART 55-102 peptide (American Peptide Co Inc, Sunnyvale, CA, a kind gift Prof. Michael J Kuhar, Emory University, Atlanta, GA, or Novo Nordisk A/S, Målöv, Denmark, kind gift Dr L. Thim), GLP-1 (Sigma Aldrich), GIP (Sigma Aldrich) (all in 10 and 100 nM concentrations) or secretagouges: glucose (5.6-25 mM) and FFAs palmitate and oleate (BSA-conjugated, 150 and 500 μM). Total RNA was isolated after 24 h and reverse-transcribed to cDNA using RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Waltham, Massachusetts). qPCR for CART, GIP, GLP-1 and two endogenous controls (PPIA and HPRT1 [29]) was performed using TaqMan Expression PCR Master Mix (Life Technologies). Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. TaqMan assays used were: CART (Mm04210469_m1), GIP (Mm00433601_m1), GCG (Mm01269055_m1), PPIA (Mm02342429_g1) and HPRT1 (Mm00446968_m1) (Life Technologies).

GLP-1 secretion assay

GLUTag cells, seeded as above, were washed twice with PBS and incubated in HEPES-buffered saline solution (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16

mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, 0.2% BSA, pH 7.2) supplemented with 0.1 mM diprotin A (Sigma Aldrich), containing 0, 2.8 or 16.7 mM glucose for 2 h. Thereafter, medium was collected for GLP-1 measurement. GLP-1 concentrations were determined using an active GLP-1 ELISA (EMD Millipore Corporation) and normalized to total protein content of each well (Bio-Rad protein assay, Bio-Rad).

Oral glucose-tolerance test (OGTT) *in vivo* in mice

Animal studies were approved by the Regional Animal Ethics Committee in Lund, Sweden. OGTT was performed after 5 h fasting in anesthetized (25mg/kg fluanison/0.8mg/kg fentanyl, Hypnorm, Veta Pharma, Leeds, UK and 12.5mg/kg midazolam, Dormicum, PanPharma, Luitre, France) female mice (22 g, C57Bl/6J, Taconic, Ejby, Denmark) with intravenous (*i.v.*) administration of 150 nmol/kg CART 54-102 or saline (0.9% NaCl). This dose was previously shown to stimulate insulin secretion during intravenous glucose tolerance test (IVGTT) in mice [17]. Glucose was introduced using oral gavage at 75g/kg 10 min after CART injection. Blood samples were collected from the retro orbital plexus at -10, 0, 10, 20, 30, 60 and 120 minutes after glucose administration and supplemented with 0.1 mmol/L diprotin A (Sigma Aldrich) and 500 KIU/mL aprotinin (Sigma Aldrich). Plasma GIP levels were determined using rat/mouse total GIP ELISA (EMD Millipore Corporation) and plasma GLP-1 levels were determined using a high sensitivity active GLP-1 ELISA (EMD Millipore Corporation).

Statistical analyses

OGTT and MMT data were analyzed using a two-way ANOVA followed by Bonferoni's multiple comparisons test and Wilcoxon signed-rank test. Protein expression was analyzed using Wilcoxon signed-rank test. All other data were

analyzed using a one-way ANOVA followed by Bonferroni´s multiple comparisons test. Differences with $p<0.05$ were considered statistically significant.

Results

CART is expressed in human K-cells and L-cells

CART immunoreactive (IR) cells were evident in the mucosal epithelium of both the duodenum and the jejunum (**Figure 1 A-C**). Double staining with chromogranin A (CgA) confirmed the endocrine identity of the CART IR cells (**Figure 1 A**). The majority of these cells were also GIP IR (75±8% in duodenum and 54±15% in jejunum**; Figure 1 D**). Of all GIP IR cells, 20±7% (duodenum) and 8±3% (jejunum) were CART IR (**Figure 1 E**). A subpopulation of the CART IR cells was also GLP-1 IR (4±4% in duodenum, **Figure 1 F** and <1%, in jejunum, data not shown). Of all GLP-1 IR cells, 16±16% in duodenum (**Figure 1 G)** and <1%, in jejunum were CART IR. Confocal microscopy revealed colocalization of CART/GIP and CART/GLP-1 in the same secretory granules (data not shown), indicating that CART may be co-secreted with both GIP and GLP-1. Since co-expression of GLP-1 and GIP has been reported [30], we triple-stained for CART, GLP-1 and GIP. This revealed that CART/GIP and CART/GLP-1 cells represented separate cell populations (data not shown).

CART plasma levels during OGTT and MMT in healthy humans

Since CART was found to be expressed in enteroendocrine cells, we hypothesized that CART is released into the circulation in response to a meal. To address this, we performed OGTT and MMT in lean healthy volunteers and measured CART plasma levels at 0, 15, 30, 60, 90 and 120 min during OGTT and at 0, 15, 30, 45, 60 and 90 min during MMT. Mean fasting CART plasma levels were 265±46 pmol/l (**Figure 2 A** and **C**). CART plasma levels displayed large individual variation and fluctuated between 28 and 875 pmol/l during the OGTT (**Figure 2 A-C**) and between 49 and 293 pmol/l during the MMT (**Figure 2 D-F**) without any apparent relation to glucose

ingestion (**Figure 2 A-C**). However, when normalization for basal levels a small increase in CART levels was apparent 60 min after the meal ($p<0.05$; **Figure 2 F**).

Regulation of CART expression by incretins, glucose and FFAs

To further study the potential biological significance of K- and L-cell CART, we next assessed whether CART is regulated by GIP and GLP-1, and stimuli for incretin secretion i.e. glucose, palmitate and oleate [31] in GLUTag and STC-1 cells; model systems for L- and K-cells respectively [32, 33].

GLP-1 had no effect on CART mRNA expression in GLUTag or STC-1 cells (**Figure 3 A** and **E** respectively). On the other hand, GIP (10 and 100 nM) increased CART mRNA levels in both GLUTag cells, (1.4±0.2-fold for 10 nM, $p<0.01$ and 1.4±0.1-fold for 100 nM, $p<0.001$; **Figure 3 A**) and STC-1 cells (1.2±0.01-fold for 100 nM GIP, $p<0.001$; **Figure 3 E**).

In GLUTag cells CART mRNA expression was unaffected by 24-h culture in 1-25 mM glucose (**Figure 3 B**). Palmitate did not affect CART mRNA levels at 5.6 mM glucose in GLUTag cells, but at 25 mM glucose, palmitate (both 150 and 500 μM) increased CART mRNA levels, compared with cells cultured at 5.6 mM glucose alone (1.60±0.33-fold, $p<0.05$ and 3.36±0.60-fold, $p<0.001$ respectively, **Figure 3 C**). Palmitate (500 μM) at 25 mM glucose also increased CART mRNA levels compared with cells cultured at 25 mM glucose alone (1.22±0.08-fold vs 3.35±0.60-fold, $p<0.001$, **Figure 3 C**). In GLUTag cells, CART mRNA levels were not affected by oleate (**Figure 3 D**).

In STC-1 cells, CART mRNA expression was also unaffected by glucose (**Figure 3 F**), but palmitate (500 μM) increased CART mRNA levels at 25 mM glucose (1.60±0.37-fold, $p<0.01$), compared with cells cultured at 25 mM glucose (**Figure 3 G).** Furthermore, at 25 mM glucose oleate (500 μM) provoked a 1.71±0.18-fold increase in CART mRNA levels compared with cells cultured in 25 mM glucose alone

($p<0.01$, **Figure 3 H**). Thus, CART mRNA expression is regulated by GIP and FFAs, but inert to GLP-1 and glucose at the concentrations tested.

Effect of CART on incretin expression and GLP-1 secretion

We next assessed the potential impact of CART on incretin expression and secretion using GLUTag and STC-1 cells as *in vitro* models. To this end, cells were treated with exogenous CART peptide or CART expression was silenced using siRNA.

Addition of CART peptide (10 nM and 100 nM for 24 h) had no effect on GIP or GLP-1 mRNA expression in GLUTag (**Figure 4 A**) or in STC-1 cells (**Figure 4 B**). Furthermore, addition of exogenous CART (10 nM) to GLUTag cells during 2 h GLP-1 secretion assay did not affect GLP-1 secretion at 0 or 16.7 mM glucose (**Figure 4 C**).

CART silencing in GLUTag cells resulted in a 82.0±0.9% reduction of CART mRNA ($p<0.001$; **Figure 4 D**), which led to a decrease in both GLP-1 mRNA expression (33.8±1.1% reduction, $p<0.001$, **Figure 4 A**) and GLP-1 content (24.9±0.3% reduction, $p<0.001$, **Figure 4 E**). Decreased GLP-1 mRNA expression after CART KD was confirmed using another siRNA (data not shown). Next, active GLP-1 secretion was assessed after 2-h static incubations, 72 h after CART silencing in GLUTag cells. CART KD had no effect on GLP-1 secretion at 0 mM glucose. However, CART-silenced cells secreted 51.1±15.7% less GLP-1 at 16.7 mM glucose compared with control cells ($p<0.01$, **Figure 4 F**).

In STC-1 cells, CART silencing led to a 62.0±3.4% reduction of CART mRNA ($p<0.001$; **Figure 4 G**). CART silencing caused 1.2±0.04-fold higher GIP mRNA expression ($p<0.001$; **Figure 4 G**). However, GIP content was not affected by CART silencing (**Figure 4 H**). We were unable to assess the effect of CART silencing on GIP secretion since the levels of GIP in the medium are below the detection limit of the ELISA. Thus, our results suggest that endogenous L-cell-derived CART exerts a

stimulatory effect on GLP-1 expression and secretion and inhibitory effect on GIP transcription.

Effect of exogenous CART on incretin secretion *in vivo*

The acute effect of exogenous CART on GIP and GLP-1 secretion was examined *in vivo* in mice (n=24 for total GIP secretion, n=21 for active GLP-1 secretion) given CART peptide (150 nmol/kg) *i.v.* during an OGTT. In the control group, GIP levels increased from 35.8±2.0 pg/ml to 534.2±65.0 pg/ml 30 min after glucose administration. In the CART-treated mice GIP levels peaked 20 min after glucose load and CART provoked an approximately 1.5±0.2-fold elevated glucose-stimulated GIP secretion at 20 min compared with saline-treated control mice ($p<0.01$, **Figure 5 A**). GLP-1 levels in control mice increased from 0.52±0.09 pM to 1.83±0.14 pM 10 min after glucose administration. CART provoked an approximately 1.5±0.2-fold increased glucose-stimulated GLP-1 secretion at 10 min ($p<0.05$, **Figure 5 B**). On the other hand, CART had no apparent effect on GIP or GLP-1 levels before administration of glucose. Thus, our results show that administration of exogenous CART elevates glucose-stimulated GIP and GLP-1 secretion *in vivo*.

DISCUSSION

Deranged incretin effect is a feature of T2D, and increased understanding of the mechanisms regulating incretin hormone secretion is important for the development of new treatment strategies. Here we show that CART is a novel constituent of human K-cells and L-cells and that CART acts as a regulator of incretin synthesis and secretion.

We found expression of CART in subpopulations of human K- and L-cells in the human upper small intestine and confocal imaging localized CART to the secretory granules in both cell types. This suggests, but does not prove, that CART is co-secreted with GIP and GLP-1. CART plasma levels were unaffected by an oral glucose load in humans. However, we observed a modest increase in CART levels 60 min after ingestion of a mixed-meal. Plasma CART levels were previously reported to be unaffected by food intake. However, CART immunoreactivity detected in the blood possibly corresponded to CART 62-102 and did not include CART 55-102 peptide that is also present in the circulation [34]. Plasma CART concentrations were in the same range in both studies [34]. It should be mentioned that CART was measured in the systemic circulation and we cannot exclude that a more pronounced postprandial response of CART in the veins draining the intestine.

CART silencing in GLUTag cells caused decreased GLP-1 secretion in response to glucose, whereas exogenous CART treatment did not have any effect on GLP-1 secretion. Although the exact mechanisms behind the effect are difficult to dissect due to the lack of identified CART receptors [6, 35], our data imply that endogenous L-cell CART exerts a stimulatory action on GLP-1 secretion. The present GLP-1 data are reminiscent of those obtained for insulin in beta-cells, in which silencing of CART caused reduced insulin secretion and production [36]. The observed reduction in insulin secretion is likely related to reduced transcription of genes encoding proteins

with important roles in the exocytotic machinery, as well as a network of beta-cell transcription factors. Thus, although further studies are needed to understand the mechanisms behind the effect of CART KD on GLP-1 secretion, it is tempting to speculate that CART has similar roles also in GLUTag cells used in the present study.

In line with the effect on GLP-1 secretion, silencing of CART caused reduced GLP-1 mRNA expression, as well as protein content. In clonal INS-1 (832/13) beta-cells CART KD resulted in decreased expression of the transcription factors *Isl1* and *Tcf7l2,* both of which have been shown to regulate GLP-1 transcription in the enteroendocrine cells [37-40]. If CART KD has the same effect in GLUTag cells, this could be a potential explanation for the observed reduction in GLP-1 mRNA expression.

The effect of CART on incretin secretion in response to an oral glucose load was studied *in vivo* in mice. In accordance with the effect of CART KD, administration of CART increased the secretion of GLP-1, but also of GIP. In mammals GIP is mainly expressed in the enteroendocrine K-cells [41]. In mice and humans, also the pancreatic alpha-cells have been reported to express GIP [44, 45]. Intestinal proGIP is mostly processed by PC1/3 to yield GIP_{1-42}, whereas in islet alpha-cells PC2 cleavage of proGIP yields the shorter form GIP_{1-30} [44]. Importantly, the islet processing form of GIP is not detected by the GIP ELISA used here. Therefore, we conclude that CART increased GIP secretion specifically from the K-cells. Although GIP mRNA expression levels do not necessarily need to be linked to the levels of hormone secretion [41], it should be noted that, silencing of CART in STC-1 cells resulted in increased expression of GIP mRNA, suggesting that endogenous K-cell CART is an inhibitor of GIP transcription. This may be a consequence of the differences in promotor architecture and binding sites for the transcription factors and factors regulating GIP and GLP-1 genes [41]. Alternatively, the stimulatory effect of CART

on GIP expression is indirect as STC-1 cells express multiple peptides, including glucagon, somatostatin, CCK and amylin, that could in turn mediate the effect on GIP expression [42, 43].

While CART increased circulating levels of GIP and GLP-1 *in vivo*, we were unable to detect any effect of exogenous CART on GIP and GLP-1 mRNA expression or GLP-1 secretion *in vitro*. There is no ready explanation for this discrepancy. The CART doses used (both *in vivo* and *in vitro*) have been shown to stimulate insulin secretion in parallel experiments [17]. This shows that the peptide is biologically active, but we cannot exclude that other doses of CART would affect GLP-1 and GIP secretion or expression in the cell lines employed. A potential explanation, could be that the stimulatory effect of CART on GIP and GLP-1 observed *in vivo* is indirect, e.g. via another hormone or via the enteric- or central nervous system.

We also studied how L- and K-cell CART is regulated by known stimulators of incretin expression and secretion. Our data show that CART mRNA expression in GLUTag and STC-1 cells was inert to ambient glucose concentration. This is different from the situation in rat pancreatic beta-cells, in which CART expression is increased by glucose [19, 46, 47]. On the other hand, CART expression was increased after culture with palmitate or oleate, depending on cell type. The doses of FFAs were at the higher end and the possibility that CART expression is increased, as part of a stress response cannot be excluded.

We also tested whether CART is regulated by GIP and GLP-1. Whereas GLP-1 was without effect, GIP increased CART expression in both cell lines. Whether CART is secreted as a consequence of elevated GIP levels remains to be established. Assessment of CART secretion into the cell media is difficult due to the rather low

CART expression levels in the cells and not enough sensitivity of the available CART assays, thus the use of *in vivo* models would be preferable.

In summary, CART is expressed in human K-cells and L-cells, and CART increases glucose-stimulated GIP and GLP-1 secretion *in vivo* in mice, and endogenous CART regulates GLP-1 and GIP expression and GLP-1 secretion in clonal L-cells. We conclude that CART is a regulator of incretin synthesis and secretion. The therapeutic potential for CART-based agents to improve incretin secretion needs further investigation.

Author Contributions

LS performed experiments, drafted and edited the manuscript; AL performed CART EIA and edited the manuscript; A-HTF performed *in vivo* experiments; EA edited and reviewed the manuscript; EZ performed confocal microscopy experiments; SEF provided human specimens and performed histological analyses; ER edited and reviewed the manuscript; JK and HH performed OGTT and MMT; NW conceived the study, drafted and edited the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Figures

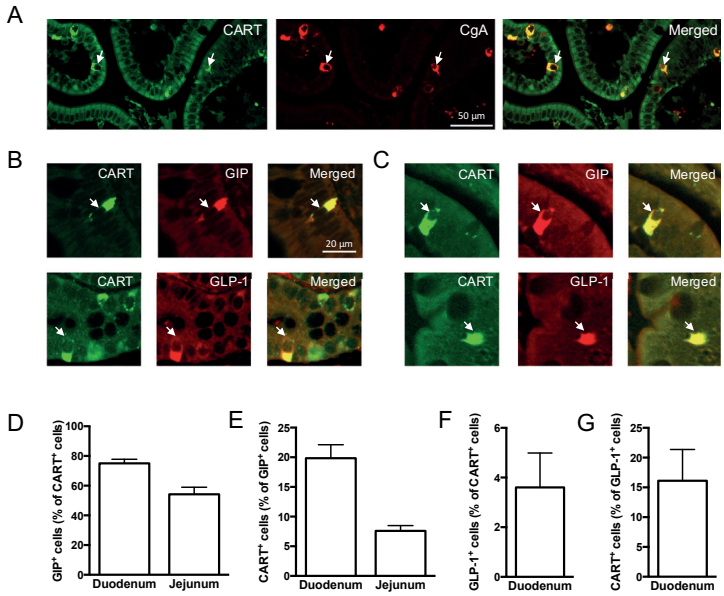

Figure 1. CART is expressed in subpopulations of human K-cells and L-cells Colocalization of CART and the endocrine cell marker chromogranin A (CgA) in human duodenal mucosa (**A**). Colocalization of CART and GIP or GLP-1 in duodenal (**B**) and jejunal mucosa (**C**). Arrows indicate colocalization. Scale bar in B for B-C. Quantification of double positive cell number relative to total number of $CART^+$ (**D**) or GIP^+cells (**E**) in duodenum and jejunum. Quantification of double positive cell number relative to total number of $CART^+$ (**F**) or $GLP\text{-}1^+$ cells (**G**) in duodenum.

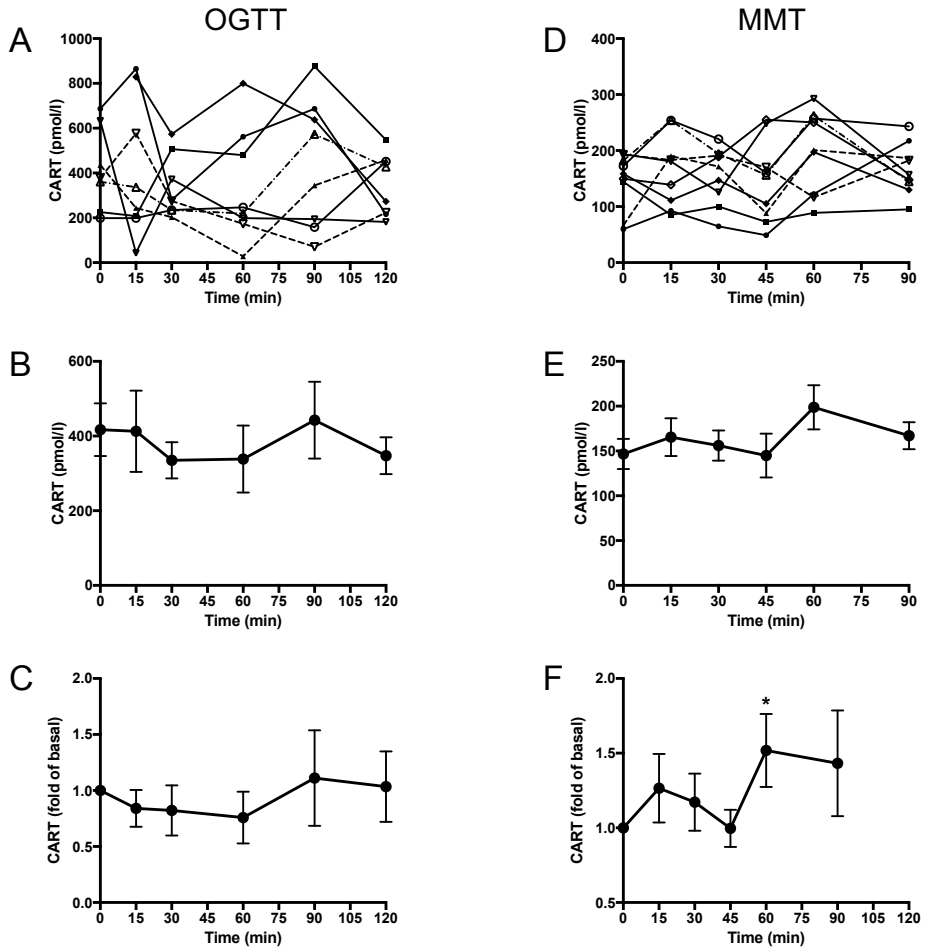

Figure 2. **CART plasma levels after an oral glucose load or a mixed-meal *in vivo* in humans**

CART plasma levels display large individual variation and seem unaffected by ingestion of 75 g glucose (**A-C**) (n=8) but are increased 60 min after a mixed-meal (**D-F**) (n=9). Each line indicates one test subject (**A and D**); data presented as mean±SEM of the CART levels (pmol/l) (**B and E**); CART levels were normalized to the basal CART concentration and presented as fold of basal (**C** and **F**), mean±SEM. *, $p < 0.05$.

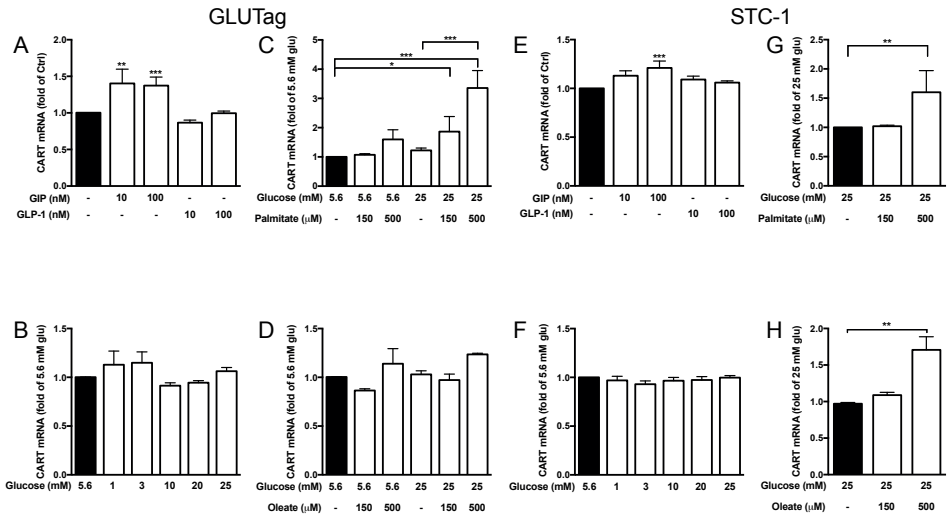

Figure 3. Regulation of CART mRNA expression in GLUTag cells and STC-1 cells
GLUTag cells (**A-D**) and STC-1 (**E-H)** cells. GIP, but not GLP-1 increases CART mRNA expression in GLUTag cells (**A**) (n=3-9). CART mRNA expression is unaffected by 24-h culture in different concentrations of glucose in GLUTag cells (**B**) (n=4). At 25 mM glucose palmitate (**C**), but not oleate (**D**) increases CART mRNA levels in GLUTag cells **(**n=3-6). In STC-1 cells, GIP, but not GLP-1 increases CART mRNA levels (**E**) (n=3-7). In STC-1 cells CART mRNA expression is unaffected by 24-h culture in different concentrations of glucose (**F**) (n=4), but palmitate (**G**) and oleate (**H**) increase CART mRNA levels **(**n=3 and n=4 respectively). Data presented as mean±SEM. *, p <0.05; **, p<0.01; ***, p<0.001.

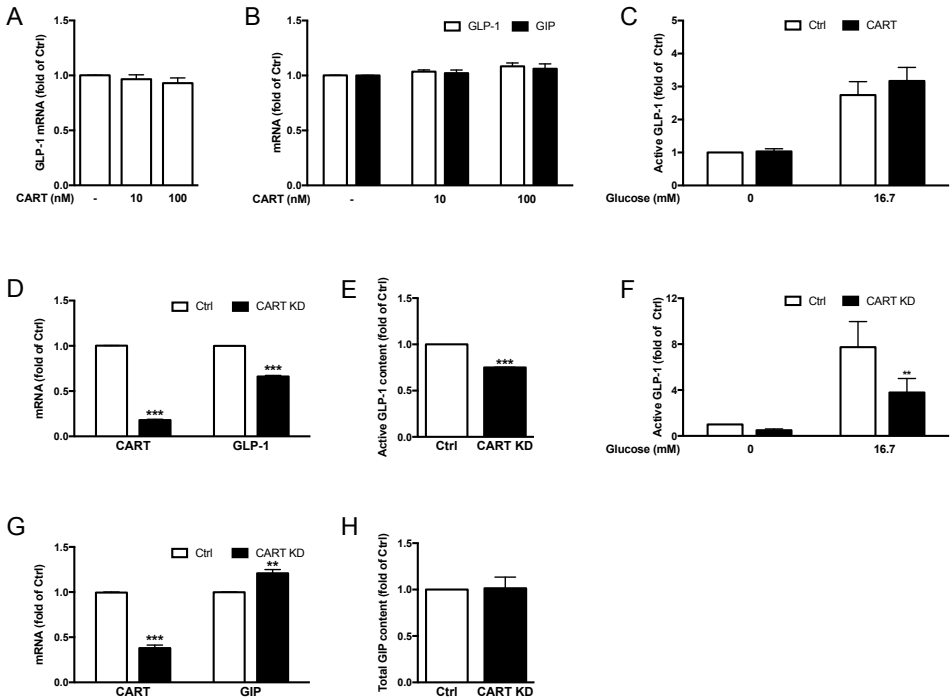

Figure 4. Effect of exogenous CART or CART silencing on incretin gene expression and GLP-1 secretion

Addition of exogenous CART does not affect incretin gene expression in GLUTag (**A**) or STC-1 (**B**) cells, and is without effect on glucose-stimulated GLP-1 secretion in GLUTag cells (**C**). CART silencing decreases GLP-1 mRNA expression (**D**) (n=3). and GLP-1 protein content in GLUTag cells (**E**) (n=4). CART silencing reduces GLP-1 secretion in response to glucose in GLUTag cells (**F**) **(**n=4). CART silencing increases GIP mRNA expression (**G**) (n=5), but is without effect on GIP protein content in STC-1 cells (**H**) (n=4). Data presented as mean±SEM. ***, $p<0.001$. Ctrl = scrambled siRNA; CART KD = CART siRNA.

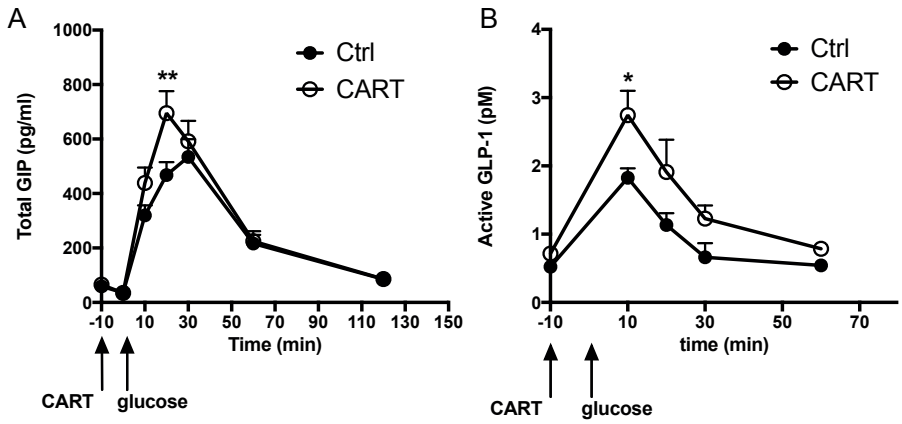

Figure 5. CART increases GIP and GLP-1 secretion during an OGTT in mice
Intravenous administration of CART caused increased circulating levels of GIP (**A**) and GLP-1 (**B**) during an OGTT (n=24 and n=21 respectively) in mice. CART was given in a tail vein at -10 min and glucose orally at 0 min as indicated by arrows. Data presented as mean+SEM. *, $p < 0.05$; **, $p < 0.01$.

Liliya Shcherbina is originally from Kazan in Russia and moved to Sweden in 2009 to perform her Master studies in cancer biology at Lund University. She achieved this goal in 2010, when she also decided to pursue a PhD. She successfully landed a PhD position at Lund University Diabetes Centre, one of the world's strongest Centers for Experimental and Clinical Diabetes research. She quickly excelled in the area, where she focused on the role of CART (Cocaine- and Amphetamine-Regulated Transcript) in insulin-producing beta-cells, as well as in intestinal K- and L-cells. Her immense efforts are collected in this Thesis, where she describes her findings in a comprehensive and mature manner.

Malin Fex, co-supervisor

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