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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, Mälöv, 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,

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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

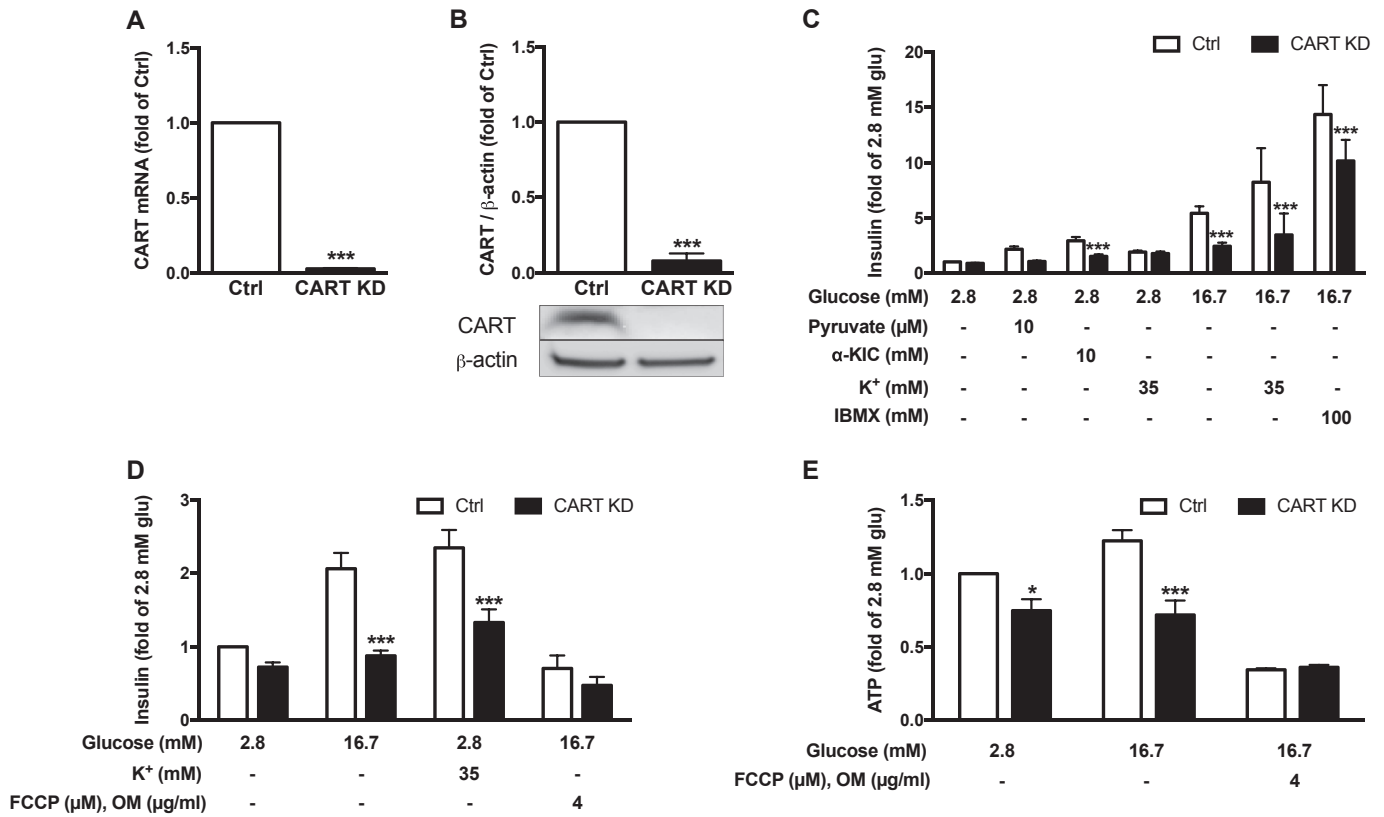

Fig. 1. CART KD reduces insulin secretion and ATP levels in beta-cells. A–B: Decreased *Cart* mRNA (A) and CART protein (B) expression after CART KD in INS-1 (832/13) cells relative to control (scrambled siRNA, Ctrl) (n = 4 and n = 6 respectively). C: CART KD reduces insulin secretion stimulated by 16.7 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. $^*p < 0.05$, $^{***}p < 0.001$.

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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

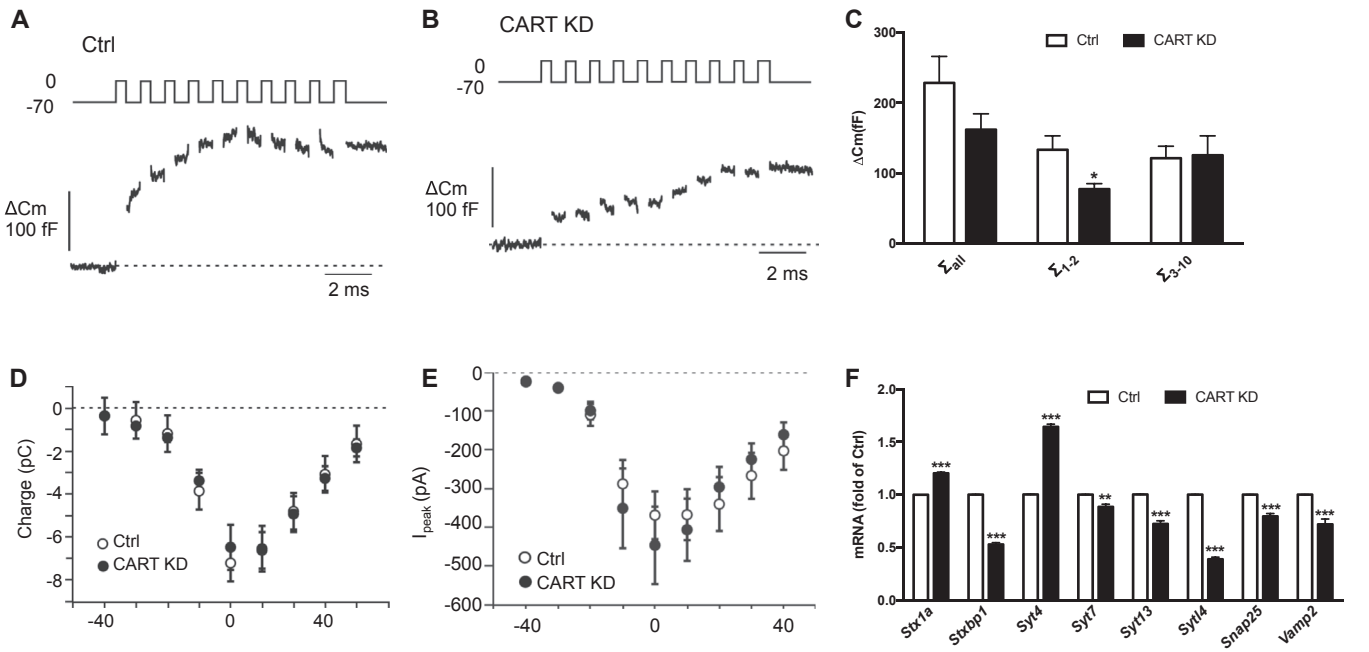

Fig. 2. CART KD reduces beta-cell exocytosis and alters expression of exocytosis genes. A–B: Representative traces of depolarization-induced exocytosis, measured as changes in cell membrane capacitance (ΔCm), in Ctrl (scrambled siRNA) (A) and CART KD (B) cells. C: Quantification of the capacitance changes induced by all depolarizations ($\sum_{all}$), by the first two depolarizations ($\sum_{1\text{-}2}$), corresponding to the readily releasable pool of granules, as well as by depolarizations 3–10 ($\sum_{3\text{-}10}$). 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). *$p < 0.05$, ***$p < 0.001$.

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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%

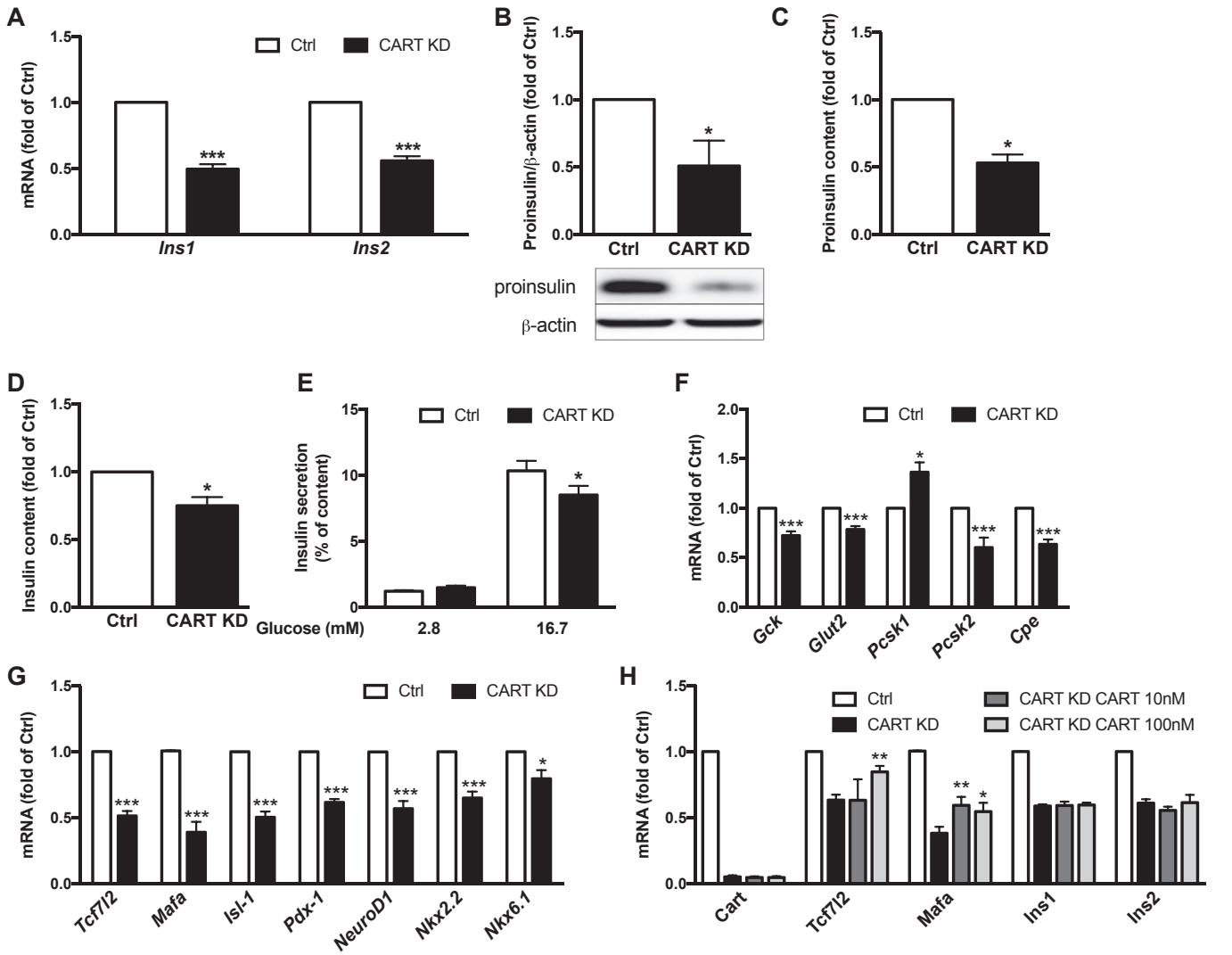

Fig. 3. CART KD decreases expression of insulin and important beta-cell genes. A: Reduced *Ins1* and *Ins2* gene expression after CART KD (n = 6) compared to Ctrl (scrambled siRNA). B: Band densitometry of Western blot and representative blot showing reduced proinsulin protein levels after CART KD (n = 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. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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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

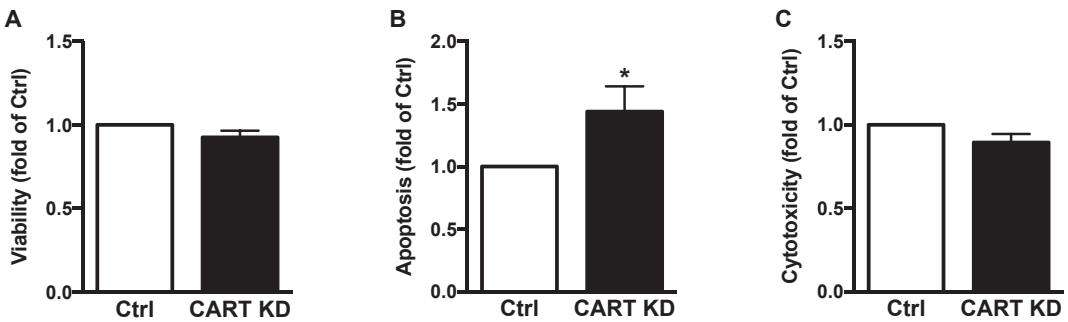

Fig. 4. CART KD increases beta cell apoptosis but does not alter viability or cytotoxicity. CART KD in INS-1 (832/13) cells results in increased apoptosis (B), without affecting cell viability (A) or cytotoxicity (C) ($n = 6$) compared to Ctrl (scrambled siRNA). Data presented as mean ± SEM. *$p < 0.05$.

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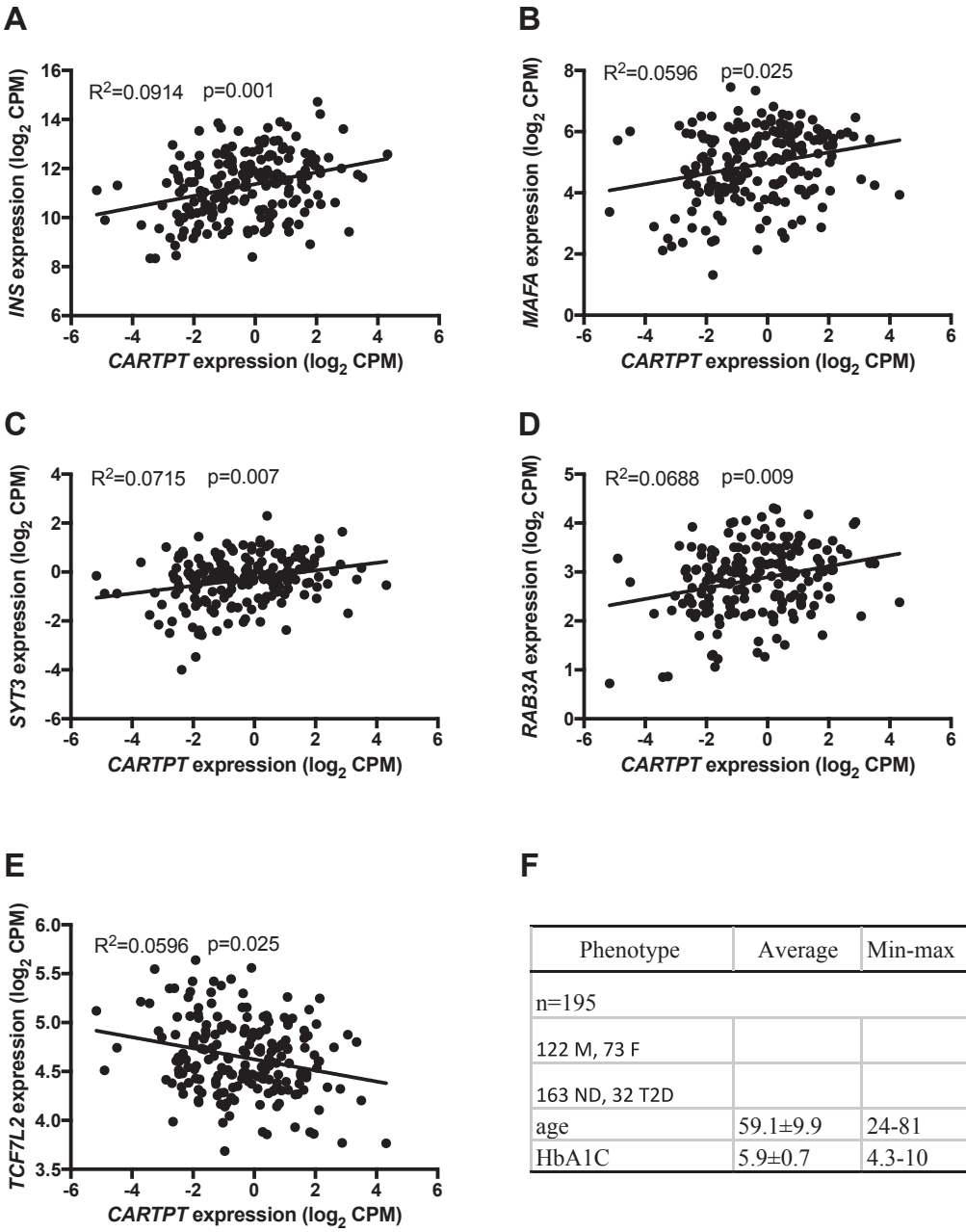

Phenotype	Average	Min-max
n=195		
122 M, 73 F		
163 ND, 32 T2D		
age	59.1±9.9	24-81
HbA1C	5.9±0.7	4.3-10

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

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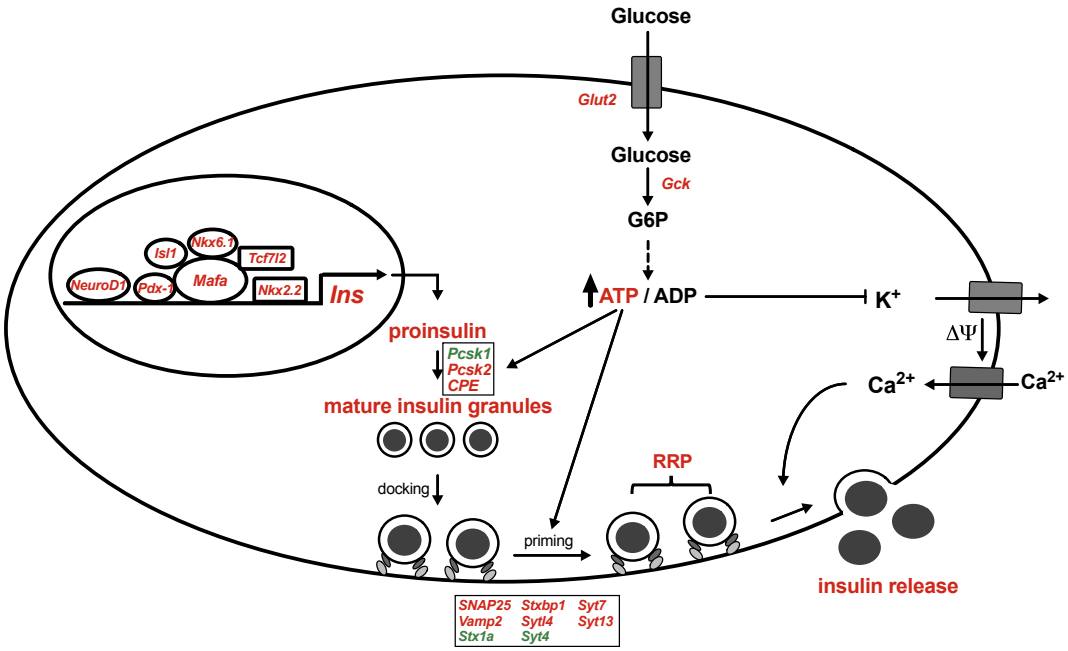

Fig. 6. Cartoon illustrating a model of how endogenous beta-cell CART regulates insulin secretion and production. Red indicates reduced, and green - increased expression or levels after CART KD.

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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