



Archived at the Flinders Academic Commons:

<http://dspace.flinders.edu.au/dspace/>

This is a copy of an article published in *Endocrinology*, and is available online at:

<http://endo.endojournals.org/content/153/11/5212.full?sid=9f9047d7-f8b7-4989-bea3-8c7c96d99061>

Please cite this as: Peiris, H., Raghupathi, R., Jessup, C.F., Zanin, M.P., Mohanasundaram, D., Mackenzie, K.D., Chataway, T.K., Clarke, J.N., Brealey, J., Coates, P.T., Pritchard, M.A., Keating, D.J., 2012. Increased expression of the glucose-responsive gene, RCAN1, causes hypoinsulinemia, beta-cell dysfunction, and diabetes. *Endocrinology*, 153(11), 5212-5221.

DOI: <http://dx.doi.org/10.1210/en.2011-2149>

© 2012 The Endocrine Society. Paper reproduced here with permission from the publisher.

Increased Expression of the Glucose-Responsive Gene, *RCAN1*, Causes Hypoinsulinemia, β -Cell Dysfunction, and Diabetes

Heshan Peiris, Ravinarayan Raghupathi, Claire F. Jessup, Mark P. Zanin, Daisy Mohanasundaram, Kimberly D. Mackenzie, Tim Chataway, Jennifer N. Clarke, John Brealey, P. Toby Coates, Melanie A. Pritchard, and Damien J. Keating

Flinders Medical Science and Technology and Centre for Neuroscience (H.P., R.R., C.F.J., M.P.Z., K.D.M., T.C., J.N.C., D.J.K.), Flinders University, Adelaide, Bedford Park SA 5042, Australia; School of Medicine (C.F.J., D.M., P.T.C.), University of Adelaide, Adelaide SA 5005, Australia; Australia Islet Consortium (D.M., P.T.C.), Institute of Medical and Veterinary Sciences (J.B.), Adelaide SA 5000, Australia; and Department of Biochemistry and Molecular Biology (M.A.P.), Monash University, Victoria 3842, Australia

RCAN1 is a chromosome 21 gene that controls secretion in endocrine cells, regulates mitochondrial function, and is sensitive to oxidative stress. Regulator of calcineurin 1 (*RCAN1*) is also an endogenous inhibitor of the protein phosphatase calcineurin, the inhibition of which leads to hypoinsulinemia and diabetes in humans and mice. However, the presence or the role of *RCAN1* in insulin-secreting β -cells and its potential role in the pathogenesis of diabetes is unknown. Hence, the aim of this study is to investigate the presence of *RCAN1* in β -cells and identify its role in β -cell function. *RCAN1* is expressed in mouse islets and in the cytosol of pancreatic β -cells. We find *RCAN1* is a glucose-responsive gene with a 1.5-fold increase in expression observed in pancreatic islets in response to chronic hyperglycemia. The overexpression of the human *RCAN1.1* isoform in mice under the regulation of its endogenous promoter causes diabetes, age-associated hyperglycemia, reduced glucose tolerance, hypoinsulinemia, loss of β -cells, reduced β -cell insulin secretion, aberrant mitochondrial reactive oxygen species production, and the down-regulation of key β -cell genes. Our data therefore identifies a novel molecular link between the overexpression of *RCAN1* and β -cell dysfunction. The glucose-responsive nature of *RCAN1* provides a potential mechanism of action associated with the β -cell dysfunction observed in diabetes. (*Endocrinology* 153: 5212–5221, 2012)

Diabetes is a metabolic disorder characterized by elevated fasting blood glucose, which arises when insulin-secreting β -cells cannot provide adequate insulin in the face of increasing insulin resistance. The pancreatic β -cell is the central regulator of glucose homeostasis and the primary source of endogenous insulin. Two major forms of diabetes exist, type 1 and type 2. Type 1 diabetes results from the autoimmune destruction of β -cells, whereas type 2 diabetes is associated with obesity, peripheral insulin resistance, and gradual insulin insufficiency. Increased levels of glucotoxicity in β -cells are thought to be central

to the pathogenesis of β -cell dysfunction in type 2 diabetes (1), but the exact nature of the molecular mechanisms underlying β -cell dysfunction in the presence of insulin resistance and hyperglycemia is unknown. Candidate genes that might trigger β -cell dysfunction under such circumstances would need to have their expression induced by hyperglycemia and have potential roles in the regulation of β -cell function.

RCAN1 is located on chromosome 21 within the q22.1–q22.2 region. The seven exons within the *RCAN1* gene give rise to two regulator of calcineurin 1 (*RCAN1*)

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Copyright © 2012 by The Endocrine Society

doi: 10.1210/en.2011-2149 Received December 19, 2011. Accepted August 20, 2012.

First Published Online September 25, 2012

Abbreviations: DAPI, 4',6-Diamino-2-phenylindole dihydrochloride; GSIS, glucose-stimulated insulin secretion; LDCV, large dense-core vesicle; *RCAN1*, regulator of calcineurin 1; ROS, reactive oxygen species; WT, wild type.

isoforms, the longer and more abundant RCAN1.1 isoform and the shorter and lowly expressed RCAN1.4 isoform. Expression of RCAN1 is highest in brain, heart, and skeletal muscle (2). RCAN1 expression is induced by a number of stress-associated stimuli including Ca^{2+} , amyloid- β and oxidative stress (3). Oxidative stress is prevalent in β -cells exposed to hyperglycemia (4), and this is due to the low expression levels of endogenous antioxidant systems in these cells.

The most well-defined function of RCAN1 is as an inhibitor of the phosphatase calcineurin (5–9), the activity of which is central to normal β -cell function and plasma insulin levels (10). Increased RCAN1 negatively affects the secretion of catecholamines from adrenal chromaffin cells (11) and mitochondrial function in neurons (12) as well as cell proliferation and tissue growth (13, 14). It is worth comparing these functions with the compromised β -cell insulin secretion, mitochondrial function, islet growth, and β -cell proliferation that are all observed in type 2 diabetes (15). Hyperglycemia-induced oxidative stress is thought to be central to β -cell dysfunction in type 2 diabetes (15), and oxidative stress induces RCAN1 expression (3). The combination of these facts suggests that aberrant RCAN1 function in β -cells may have implications in the pathogenesis of β -cell failure in type 2 diabetes. Based on its known roles in regulating secretion, mitochondrial function, calcineurin activity, and cell proliferation, we investigated the role of RCAN1 in β -cell function. Our findings demonstrate that the overexpression of RCAN1 in mice causes diabetes characterized by hypoinulinemia and β -cell dysfunction via multiple pathways.

Materials and Methods

Animals

RCAN1 transgenic (RCAN1^{ox}) mice were generated using human RCAN1 cDNA encoding the exon 1 splice variant as described (11). The minigene transgene construct consisted of a 4-kb region up-stream of exon 1 and also contained exons 5–7 and their flanking introns. The minigene DNA was prepared for microinjection by a double digestion with *HindIII* and *NotI*, and the gel-purified insert was used to microinject fertilized mouse embryos of C57:B6 \times CBA genetic background. The gene construct was under the regulation of its endogenous promoter. The wild-type (WT) mice were of C57:B6 \times CBA genetic background. WT and RCAN1^{ox} mice were maintained in the Flinders University Animal House under pathogen-free conditions in a 12-h light, 12-h dark cycle, and use was approved by the Flinders University Animal Welfare Committee. Male mice were used in all studies.

Fasting blood glucose measurement

Fasting blood glucose levels were measured at the ages indicated. Before measurement, mice were transferred to a fresh cage without food for 16 h. All mice had access to water *ad libitum* during this period. Samples were obtained at the ages indicated. Blood was sampled from the tail, and fasting blood glucose levels were measured using an ACCU-CHEK Performa glucometer (Roche Diagnostics, Castle Hill, Australia).

Isolation of mouse pancreatic islets

Mice were killed by an anesthetic overdose of isoflurane and islets isolated as previously described (16).

Quantitative real-time PCR

RNA was extracted from mouse islets using the RNeasy Mini Kit (QIAGEN, Doncaster, Australia). Real-time PCR was carried out using the QIAGEN Quantitect SYBR Green PCR kit (QIAGEN, Australia) and RotorGene 3000 thermocycler (Corbett Life Science, Sydney, Australia). All real-time experiments were carried out using 3 μl of diluted cDNA. The *18s* rRNA primer sets were used as the housekeeping gene to normalize samples and evaluate changes in expression. A sample with a no-cDNA template was the negative control. All samples were run in triplicate. Primer sequences and other information for all target genes are provided in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>).

Immunoblot analysis of RCAN1

Immunoblot analysis of RCAN1 was performed using anti-RCAN1 antibodies at a dilution of 1:200 (Sigma-Aldrich, St. Louis, MO). Proteins were extracted in RIPA buffer containing 0.1% SDS using a tissue-lyser (QIAGEN, Australia). Protein concentration was estimated using the EZQ assay (Invitrogen, Australia). 25 μg of each sample was separated on a Criterion TGX stain-free gel (Bio-Rad, Australia) and transferred onto 0.2- μm polyvinylidene fluoride membrane (Roche Applied Science, Australia). Blots were probed using a donkey antirabbit-horseradish peroxidase-conjugated secondary antibody at a dilution of 1:100 (Thermo Scientific) and West Pico (Pierce) as the substrate for chemiluminescent detection. Blots were visualized on a Fujifilm LAS4000 and Gel Doc Ez-Imagers (Bio-Rad, Australia). Densitometry was performed with Carestream Molecular Imaging Software (Carestream Health).

Insulin measurements

Blood insulin levels were measured after 16 h fasting using an ultrasensitive mouse insulin ELISA kit (Crystal Chem Downers Grove, IL) according to the manufacturer's instructions for a low-range assay (17). Absorbance was measured at 450 and 620 nm using a BIOMECK-3000 microplate reader (Beckman-Coulter Brea, CA) and MultiMode detection software. For *in vitro* glucose-stimulated insulin secretion (GSIS), 10 islets were incubated in 50 μl of Krebs buffer (supplemented with 3 mM glucose) at 37 C for 1 h (basal secretion). Islets were exposed to Krebs buffer with 20 mM glucose for 10 min (first-phase GSIS) and 50 min (second-phase GSIS). Islets were allowed to recover for 1 h at 3 mM glucose before being exposed to a high- K^+ (70 mM) Krebs solution for 20 min. Samples were centrifuged at 250 $\times g$ for 1 min, the supernatant removed, and insulin content

measured as described previously. To measure islet insulin content, 10 islets were subjected to 12 freeze-thaw cycles in RIPA buffer and centrifuged at $250 \times g$ for 1 min. The supernatant was diluted 1:1000 in PBS and insulin content measured by ELISA.

Glucose tolerance test

Glucose tolerance tests were carried out on mice fasted for 16 h. Glucose (2 mg/g body weight) (PharmaLab, Australia) was injected (ip) into mice and blood glucose measured at 15-min intervals from 0–240 min via a tail bleed using an ACCU-CHEK Performa glucometer (Roche Diagnostics, Australia).

Immunohistochemical labeling in pancreatic sections and MIN6 cells

Whole pancreatic tissue was fixed in 10% buffered formalin for 24 h after excision from the animal. Tissue was then washed in PBS (pH 7.2) supplemented with sodium azide for 2 h and then washed in running deionized water for 2 h. The tissue was subsequently dehydrated in ethanol (80, 90, and 100% for 2 h in each solution) and then transferred to chloroform overnight. The tissue was embedded in Paraplast wax for 1 h (62 C) and fresh wax for 1.5 h and finally embedded in fresh wax. The 5- μ m sections were allowed to dry overnight in a 37 C oven. Guinea pig polyclonal antibody to insulin (AbCam, Cambridge, MA) was used diluted 1:3200 in antibody diluent [NaCl, Na₂HPO₄, Na₂HPO₄ 2H₂O, 10% NaN₃ in distilled water (pH 7.1)]. Biotin-streptavidin-conjugated donkey anti-guinea pig secondary antibody (Jackson ImmunoResearch, West Grove, PA Laboratories) was used at a dilution of 1:200 and labeled with Cy3-conjugated streptavidin (Jackson Laboratories), and 3 μ M 4',6-diamino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, Australia) was used as a nuclear marker.

MIN6 β -cells were grown for 24 h on glass coverslips coated with 10 μ g/ml poly-D-lysine and 10 μ g/ml laminin (Sigma-Aldrich) and in DMEM containing 10% FBS, 1% penicillin-streptomycin (1 mg/ml), 1% L-glutamine, and 5 μ l/liter β -mercaptoethanol (Sigma-Aldrich). Cells were fixed for 18–20 h in Zamboni's fixative at 4 C followed by washes in 80% ethanol (four times for 5 min each), 100% ethanol (twice for 5 min each), dimethylsulfoxide (three times for 5 min each), and PBS (four times for 5 min each). Fixed cells were incubated for 30 min in 10% normal donkey serum, placed for 24 h at room temperature in a humidifier with rabbit monoclonal antibody against RCAN1 (Sigma-Aldrich) (final dilution 1:10) and guinea pig monoclonal antibody against insulin (Abcam) (final dilution 1:3200). The cells were washed with PBS and incubated in donkey antirabbit IgG-Cy5 (Jackson ImmunoResearch) (final dilution 1:400) and donkey anti-guinea pig IgG tagged with Cy3 and DAPI as described in the previous paragraph. After washing with PBS, the coverslips were mounted onto glass slides in buffered glycerol and fluorescence visualized on a Leica TCS SP5 Spectral confocal microscope.

Measurement of islet size and β -cell density

Islet size was determined by measuring the insulin-positive area of individual islets in paraffin embedded pancreatic sections. β -cell density of an islet was measured by counting the number of DAPI-positive cells per islet area. Analysis was carried out on ImageJ image analysis software. 8–10 islets were measured from each animal.

Insulin tolerance test

Insulin tolerance tests were carried on mice fasted for 1 h, and 0.75 or 1 U/kg body weight insulin (Novo Nordisk, Australia) was injected (ip) into 40- and 120-d-old mice, respectively. Blood glucose was measured via a tail bleed from 0–120 min at 15-min intervals using an ACCU-CHEK Performa glucometer (Roche Diagnostics, Australia).

Islet reactive oxygen species (ROS) production

After isolation, islets were allowed to recover overnight in phenol red-free RPMI (10% heat inactivated fetal calf serum). Islets were transferred into media supplemented with 20 or 5 mM glucose (control) for 24 h. Islets were incubated with 5 μ M MitoSox Red (Invitrogen, Australia) for 10 min, washed twice in Krebs solution, and visualized on an IX-71 fluorescent microscope (Olympus, Japan). Mean fluorescence intensity of individual islets was calculated using ImageJ image analysis software. Seven to 10 islets were measured from each animal.

Electron microscopy studies

The pancreas was cut into cubes of approximately 0.5 mm³ and processed for electron microscopy as reported (18). Sections were examined using a Hitachi H-600 transmission electron microscope (Tokyo, Japan). β -Cells were distinguished from α - and δ -cells by the presence of their characteristic granules as described in previous studies (18). Insulin secretory vesicle density was calculated by counting the number of large dense-core vesicles (LDCVs) per cell and normalized to cell area. To measure vesicle localization, 200-nm-wide concentric zones starting from the cell membrane were defined within each β -cell and the number of dense core vesicles within each region counted and presented as a percentage of the total number of vesicles within each cell. Filled secretory vesicles containing insulin were clearly identified by the presence of a dense core within the vesicle. Empty secretory vesicles were identified as being a membrane-bound vesicle in the range of 100–300 nm in diameter, the same size range as that found for filled vesicles. All analysis was carried out on $\times 4000$ electron micrographs using ImageJ image analysis software.

Statistical analysis

Parametrically distributed data were analyzed using an unpaired Student's *t* tests and for nonparametric data sets a Mann-Whitney *U* test was used. Statistical significance was $P < 0.05$. All data are shown as mean \pm SEM.

Results

RCAN1 is a glucose-responsive gene present in β -cells that regulates the expression of islet gene products

To study the role of RCAN1 in β -cell function, we confirmed the presence of the *RCAN1* gene in mouse pancreatic islets and MIN6 β -cells (Fig. 1A). Using immunohistochemistry, we also observed a largely cytoplasmic expression of RCAN1 protein in MIN6 β -cells (Fig. 1B). Chronic high glucose is thought to induce oxidative stress

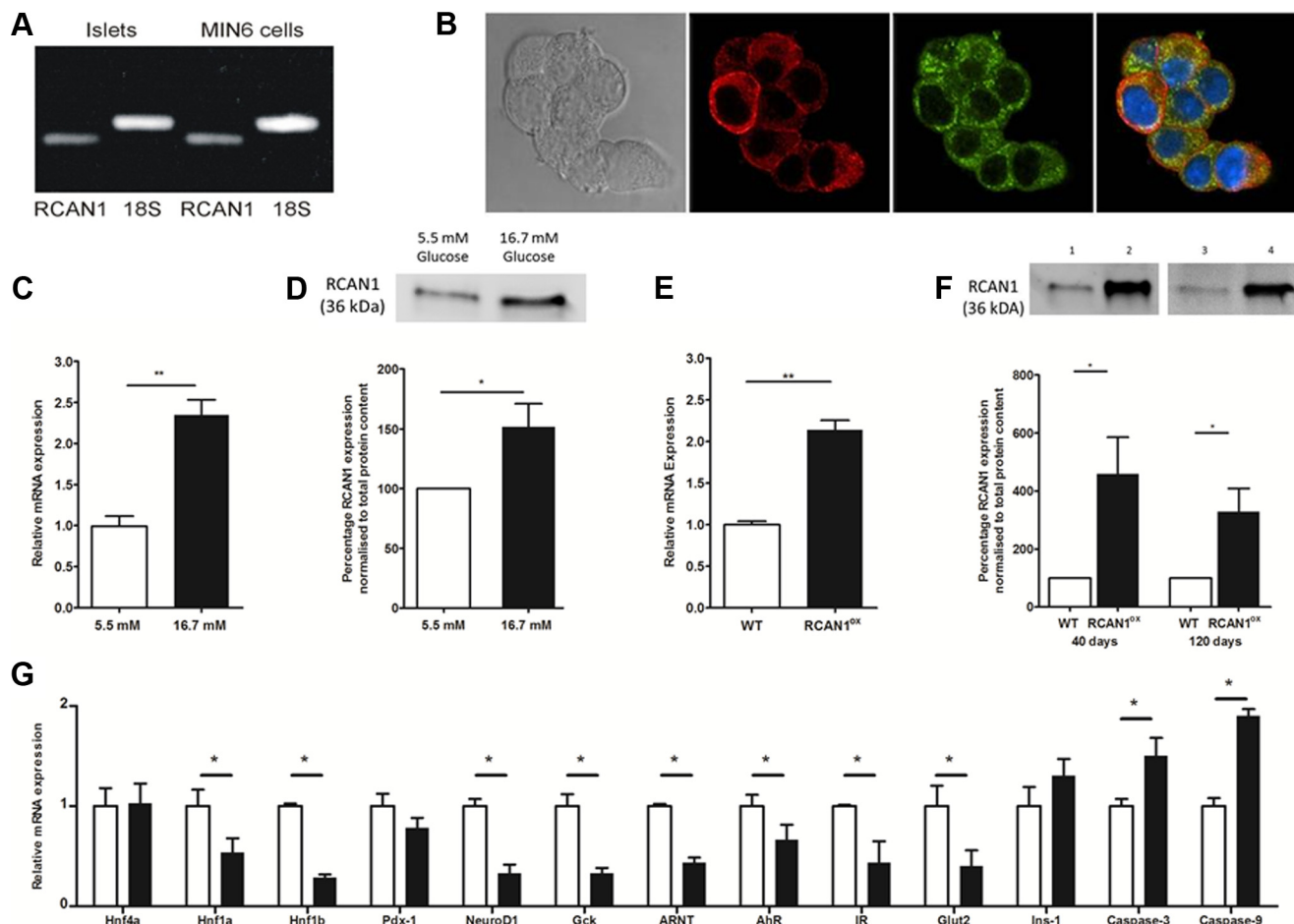


FIG. 1. RCAN1 is expressed in β -cells where it is responsive to hyperglycemia and regulates the expression of essential β -cell gene products. A, RCAN1 is endogenously expressed in mouse islets and β -cells. PCR using mRNA isolated from islets and the MIN6 β -cell line and primers specific to exon 7 of the RCAN1 genome illustrates RCAN1 expression. B, RCAN1 protein expression is cytoplasmic in mouse MIN6 β -cells; from *left to right*, bright-field image of MIN6 cells, insulin (red), RCAN1 (green), and merged image of insulin, RCAN1, and DAPI (blue). C and D, Real-time RT-PCR (C) and Western blot analysis (D) in WT islets illustrates that chronic (6 d) hyperglycemia induces islet RCAN1 expression. E, Real-time RT-PCR was performed on isolated islets from 120-d-old WT and RCAN1^{ox} mice. F, Western blot analysis illustrates RCAN1 expression in WT islets and RCAN1 overexpression in RCAN1^{ox} islets at 40 d (lane 1 and 2) and 120 d (lane 3 and 4). G, Real-time RT-PCR was performed on isolated islets from WT and RCAN1^{ox} mice for β -cell gene products. White bars, WT; black bars, RCAN1^{ox} (n = 5 animals per genotype). *, $P < 0.05$.

in β -cells, leading to β -cell dysfunction and β -cell loss in type 2 diabetes. Because oxidative stress induces RCAN1 expression, we hypothesized that chronic high glucose exposure would induce β -cell RCAN1 expression. Using real-time PCR (Fig. 1C) and Western blotting (Fig. 1D), we found that *in vitro* islet RCAN1 expression is induced by chronic (6 d) hyperglycemia. To study the effect of RCAN1 overexpression on glucose homeostasis and β -cell function, we used mice in which human RCAN1.1 is overexpressed (RCAN1^{ox} mice) (11). Overexpression of RCAN1 in these mice occurs only in cells in which RCAN1 is endogenously expressed. These mice are viable and fertile and display no overt phenotype. Blood analysis indicates no change in markers associated with liver or kidney function or in cholesterol, triglyceride, or high-density lipoprotein levels (data not shown) when RCAN1^{ox} mice are compared with controls. The overexpression of

RCAN1.1 was confirmed in pancreatic islets of wild-type and RCAN1^{ox} mice (Fig. 1E). RCAN1 protein levels in pancreatic islets of RCAN1^{ox} mice at 40 and 120 d of age also confirmed the overexpression of RCAN1.1 (Fig. 1F). Because the calcineurin/nuclear factor of activated T-cells pathway is a known regulator of β -cell gene expression (10) and RCAN1 inhibits calcineurin activity, we tested whether important β -cell genes are regulated by RCAN1 overexpression. We identified multiple gene targets down-regulated in RCAN1^{ox} islets (Fig. 1G). These include *Hnf1a*, *Hnf1b*, *NeuroD1*, and glucokinase, mutations in which cause monogenic forms of type 2 diabetes known as maturity-onset diabetes of the young (MODY). These are each regulated by the calcineurin/nuclear factor of activated T-cells pathway (10). Additionally, the expression of the transcription factor *ARNT* and its cofactor *Ahr*, implicated in β -cell dysfunction in type 2 diabetes (19), are

both reduced in RCAN1^{ox} islets. The expression of genes encoding the glucose transporter 2 and the insulin receptor are also reduced. *Ins-1* expression is not altered, in agreement with the lack of change in total insulin content we observe in RCAN1^{ox} islets (see Fig. 4B). We also observe an increased expression of the apoptotic markers caspase-3 and caspase-9 in RCAN1^{ox} islets (Fig. 1G).

RCAN1^{ox} mice develop diabetes

RCAN1^{ox} mice develop increasingly severe hyperglycemia and overt diabetes from 60 d of age compared with age-matched WT controls (Fig. 2A). This is not due to increased body weight in RCAN1^{ox} mice (Fig. 2B). Glucose tolerance was assessed by ip glucose tolerance tests at two different ages, before and after the onset of hyperglycemia in RCAN1^{ox} mice. Glucose tolerance was unchanged at 40 d (Fig. 2C), but RCAN1^{ox} mice demonstrate impaired glucose tolerance at 120 d (Fig. 2D). The hyper-

glycemia observed in RCAN1^{ox} mice is likely caused either by increased insulin resistance or by decreased plasma insulin levels. To identify whether such changes occur, we measured insulin resistance in RCAN1^{ox} mice. No significant difference in blood glucose between WT and RCAN1^{ox} mice was observed at any of the time points in response to the injected insulin at both 40 d (data not shown) and 120 d of age (Fig. 2E). However, when we transform these results into relative changes in insulin-induced blood glucose (Supplemental Fig. 1), we see that RCAN1^{ox} mice have increased sensitivity to an exogenous insulin load at 120 d. Thus, RCAN1^{ox} mice may develop an increased response to a given amount of insulin as they age, and RCAN1^{ox} mice clearly do not develop any insulin resistance. The absence of insulin resistance in RCAN1^{ox} mice suggests that hypoinsulinemia may underlie the age-associated diabetes observed in RCAN1^{ox} mice. Consistent with this, circulating insulin levels were significantly lower at 120 d in RCAN1^{ox} mice (Fig. 2F).

RCAN1 regulates islet morphology and β -cell number

To identify whether this reduction in circulating insulin could be caused by a loss of β -cells, we stained pancreatic sections for insulin (Fig. 3A) and measured total β -cell mass per islet. Islet size was significantly smaller in RCAN1^{ox} mice at 100 d ($P < 0.001$) but not at 40 d (Fig. 3B). Islet shrinkage was due to a reduction in total β -cell number per islet (Fig. 3C) rather than decreased β -cell size (Fig. 3D).

RCAN1 regulates insulin secretion and insulin loading in β -cells

Given the reductions in circulating insulin levels and β -cell number in RCAN1^{ox} mice, we next wished to identify whether elevated RCAN1 expression affects insulin secretion. Static *in vitro* islet insulin secretion (normalized to total islet protein content to compensate for variations in islet size) was measured to gauge β -cell secretory capacity. Basal ($P < 0.05$) and first-phase (10 min, $P < 0.05$) GSIS were both reduced in RCAN1^{ox} islets, whereas second-phase secretion (10–60 min after glucose stimulation) was not altered (Fig. 4A). The reduction

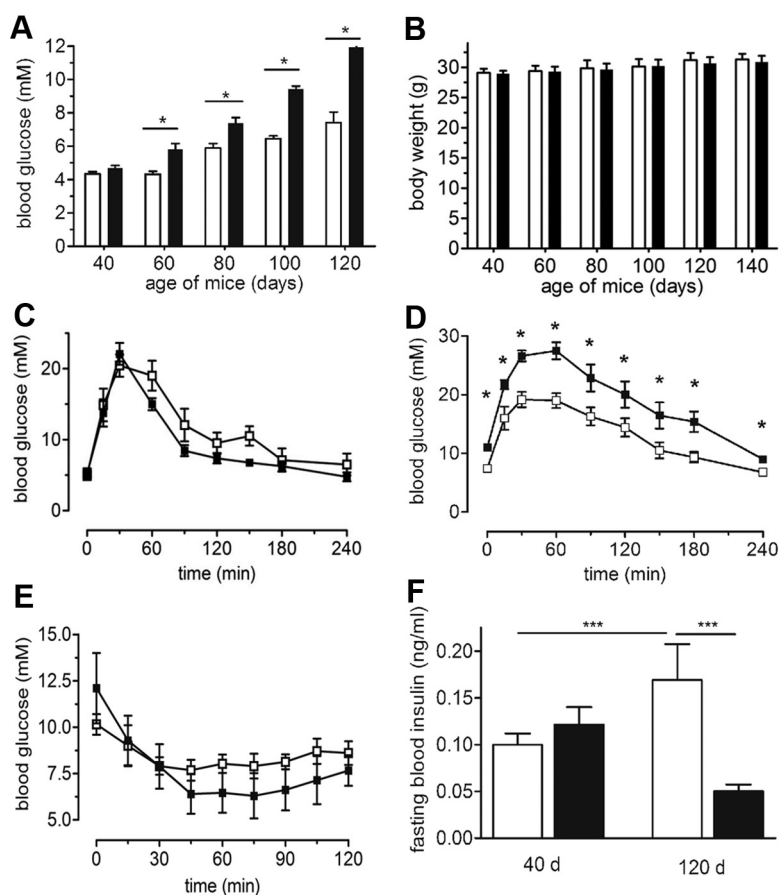


FIG. 2. RCAN1^{ox} mice develop diabetes. A, RCAN1^{ox} mice (black bars) have higher fasting blood glucose levels from 60 d compared with WT controls (white bars). B, Body weight is similar in WT (white bars) and RCAN1^{ox} mice (black bars) at all ages. C and D, Glucose tolerance is similar between groups at 40 d (C) but is reduced in RCAN1^{ox} mice at 120 d of age (D) ($n = 5–10$ animals per genotype). *, $P < 0.05$. White squares, WT; black squares, RCAN1^{ox}. E, The 120-d-old mice were injected ip with insulin (1 U/kg body weight) and blood glucose measured at times indicated. No differences in glucose level were observed between the two groups. F, Fasting blood insulin is similar at 40 d but significantly lower in RCAN1^{ox} mice at 120 d.

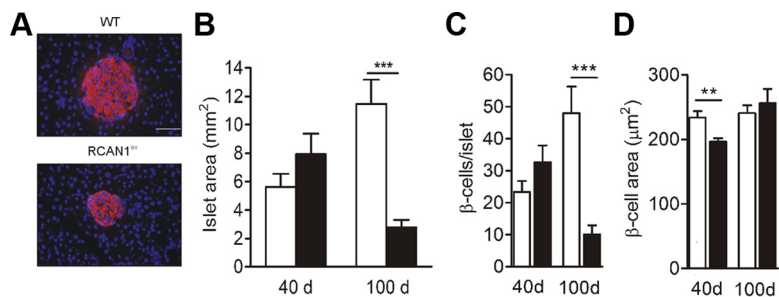


FIG. 3. RCAN1 regulates islet morphology and β -cell number. A, Pancreatic sections were labeled with an antiinsulin antibody (red) and nuclear marker DAPI (blue) to measure islet size. Scale bar, 50 μ m. B–D, Islet size was similar between groups at 40 d but significantly smaller in RCAN1^{ox} mice at 100 d (B) because there are fewer β -cells per islet in RCAN1^{ox} mice (C) and not due to any change in β -cell size at 120 d as measured from electron microscopy images (D). *, $P < 0.05$; ***, $P < 0.001$; $n = 5$ –9 animals per genotype. Black bars, RCAN1^{ox} mice; white bars, WT mice.

in secretory capacity was not due to decreased insulin content within pancreatic islets (Fig. 4B). Electron microscopic analysis of β -cells was carried out to investigate

Because RCAN1 overexpression caused an increase in empty vesicles, we hypothesized that the filling of LDCVs

might be negatively affected in RCAN1^{ox} β -cells. We detected a small (~8%) but significant reduction in the diameter of the dense core (containing insulin) within LDCVs in RCAN1^{ox} β -cells (111.0 ± 1.6 nm) compared with WT β -cells (120.8 ± 1.8 nm, $P < 0.01$) (data not shown), indicating reduced insulin loading into RCAN1^{ox} LDCVs. This is not due to a concomitant reduction in total LDCV diameter in RCAN1^{ox} β -cells (data not shown).

RCAN1 affects mitochondrial morphology and function

Mitochondrial changes and increased ROS production are observed in β -cells from type 2 diabetes patients (15) and upon overexpression in neurons of the *Drosophila melanogaster* RCAN1 ortholog nebula (12). The ultrastructural appearance of mitochondria is unchanged in RCAN1^{ox} β -cells (Fig. 5A). Although the same number of mitochondria were seen in each group (Fig. 5B), mitochondrial size was reduced in RCAN1^{ox} β -cells ($P < 0.05$, Fig. 5C). Hyperglycemia-induced oxidative stress reduces mitochondrial size (20). We therefore measured mitochondrial ROS production in WT and RCAN1^{ox} islets using the mitochondrial ROS marker MitoSox (Fig. 5D). Basal (3 mM glucose) mitochondrial

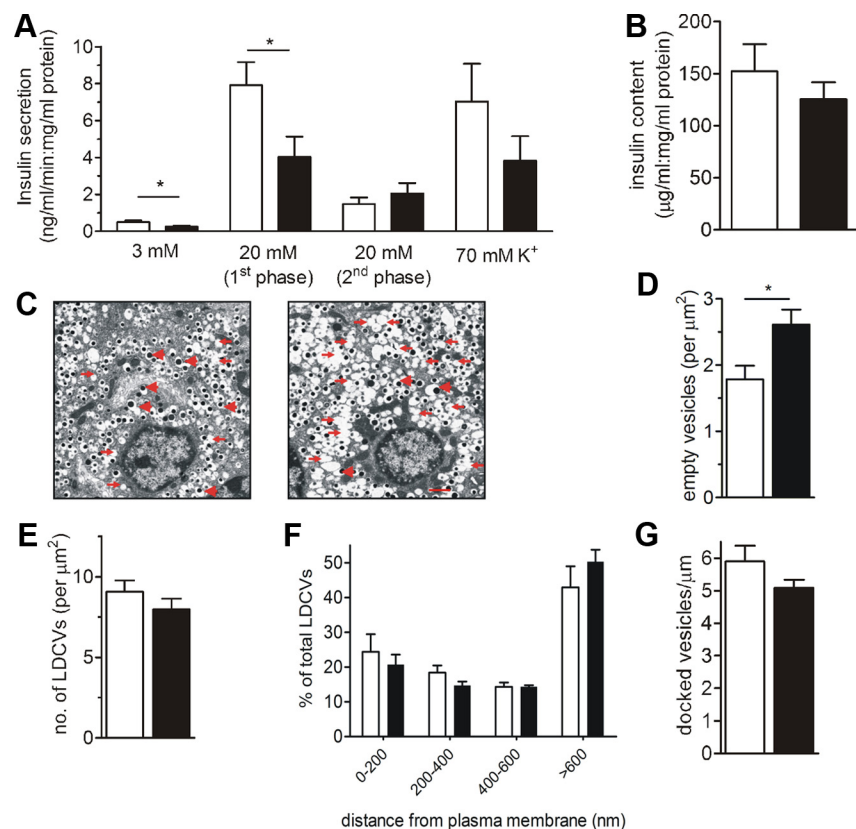


FIG. 4. RCAN1 regulates insulin secretion and vesicle loading in β -cells. A, Insulin secretion is compromised in RCAN1^{ox} β -cells. Basal (3 mM glucose for 1 h) and first-phase (time = 0–10 min in 20 mM glucose) insulin secretion are significantly reduced in RCAN1^{ox} islets, whereas second-phase (time = 10–60 min in 20 mM glucose) secretion is not altered ($n = 5$ mice sampled in triplicate per group). B, Total insulin content of islets is unaltered ($n = 13$ –15). C, Electron micrographs of a WT (left) and RCAN1^{ox} (right) β -cell demonstrate the presence of both filled and empty (arrows) secretory vesicles within the cytosol of β -cells. D and E, The number of empty secretory vesicles (D) but not filled vesicles (E) is increased in RCAN1^{ox} β -cells. F and G, We did not observe differences in the proximity of insulin-containing vesicles to the plasma membrane (F) or the number of docked vesicles (G) ($n = 3$ –5 animals and 6–8 β -cells per genotype). *, $P < 0.05$. White bars, WT; black bars, RCAN1^{ox}. Scale bar, 500 nm (C).

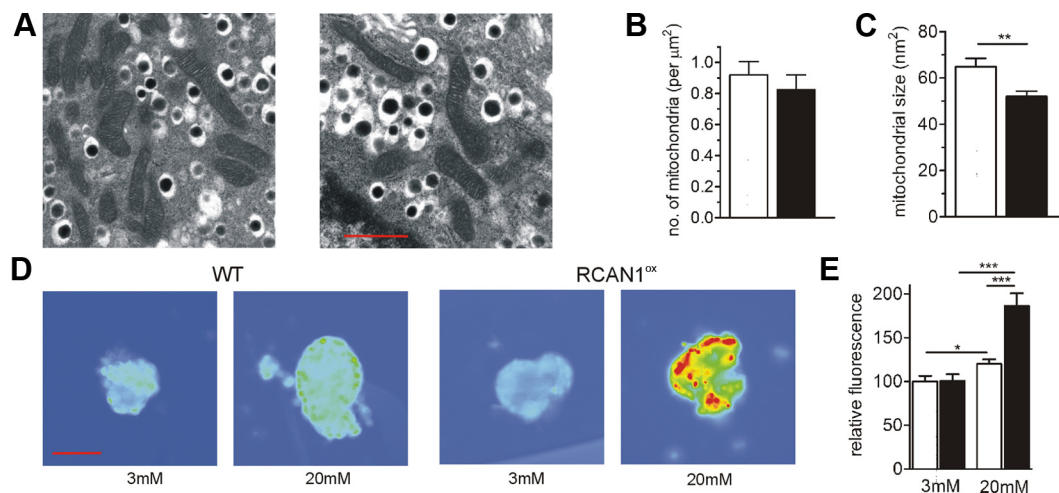


FIG. 5. RCAN1 regulates mitochondrial size and ROS production. A, Electron micrographs of WT (*left*) and RCAN1^{ox} (*right*) β -cell mitochondria illustrating similar mitochondrial appearance. B and C, Mitochondrial density (B) is similar, whereas mitochondrial size (C) is smaller in RCAN1^{ox} β -cells ($n = 3$ animals per genotype, $n = 6$ – 8 β -cells and 170–208 mitochondria). D, MitoSox fluorescence in WT and RCAN1^{ox} islets under various conditions. E, Mitochondrial ROS production is greater in response to 20 mM glucose in RCAN1^{ox} islets; $n = 3$ individual islet preparations and 20–32 islets. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Scale bar, 500 nm (A) and 200 μm (D).

ROS production in islets was similar between both groups (Fig. 5E). After exposure to 20 mM glucose, WT islets displayed moderately increased mitochondrial ROS production ($P < 0.05$), consistent with previous reports (4). However glucose-induced increase in mitochondrial ROS production was greater in RCAN1^{ox} islets ($P < 0.001$, Fig. 5E), illustrating RCAN1-induced mitochondrial dysfunction.

Discussion

Normal β -cell function and insulin secretion are essential for the regulation of circulating plasma glucose levels. This current study identifies the RCAN1 gene as being involved in the proper control of glucose homeostasis due to its effect on multiple facets of β -cell function. We further identify RCAN1 as a glucose-regulated gene that has increased expression in islets under glucotoxic conditions. We demonstrate that when RCAN1 expression is increased *in vivo* in mice, the result is the development of diabetes associated with β -cell failure. In RCAN1^{ox} mice, age-dependent hyperglycemia, impaired glucose tolerance, hypoinsulinemia, reduced β -cell secretion, reduced β -cell number, decreased insulin granule filling, aberrant mitochondrial ROS production, and the altered expression of key β -cell genes occur. Thus, RCAN1 is an important β -cell gene, regulating multiple facets of β -cell function.

The experiments used mice in which RCAN1 is over-expressed in all cells that normally express RCAN1. Hence, the interpretations of our work cannot be solely confined to effects of RCAN1 directly on β -cells. Al-

though studies have shown the presence of RCAN1 in the exocrine acinar cells of the pancreas (21), it is currently unknown whether RCAN1 is present in the endocrine α - and δ -cells of the pancreatic islet. Furthermore, we observed no overt changes in liver or kidney function or changes in blood lipids or in body weight. These results also demonstrate that RCAN1^{ox} mice are not insulin resistant but rather show an increased response to insulin at 100 d. What underlies this increase in insulin sensitivity is unknown. It may be due to an undescribed role of RCAN1 in regulating insulin sensitivity in peripheral tissues in which it is expressed, such as skeletal muscle or liver (2). Alternatively, this change in insulin sensitivity may reflect a physiological adjustment by RCAN1^{ox} mice in response to the lower levels of circulating insulin to maintain glucose homeostasis and minimize hyperglycemia. Many of our *in vitro* studies were also performed using isolated islets to alleviate concerns regarding non- β -cell effects of elevated RCAN1 expression. These islets were maintained in culture for several days before experimentation, potentially removing any indirect *in vivo* effects that could potentially occur in RCAN1^{ox} mice. This tissue was obtained at an age in which these mice are hyperglycemic, and there is the additional possibility that hyperglycemia may be driving some of the β -cell changes we observed, such as aberrant ROS production, gene expression changes, and reduced insulin secretion. If *in vivo* hyperglycemia was still affecting *in vitro* islet function, however, higher resting ROS levels would have been observed in RCAN1^{ox} islets. Because we do not observe such changes, we are confident that the 72- to 96-h culture period used in our *in vitro*

experiments is sufficient to remove potential confounding effects of hyperglycemia in RCAN1^{ox} islets.

The diabetes associated with RCAN1 overexpression is due to hypoinsulinemia caused by β -cell loss and dysfunction. One mechanism associated with this β -cell loss may be increased apoptosis, as indicated by the elevated expression of the apoptosis gene markers caspase-3 and caspase-9 in RCAN1^{ox} islets. Overexpression of RCAN1.1 in primary neurons activates both caspase-9 and caspase-3, which induces neuronal apoptosis (22). This neurotoxic effect of RCAN1.1 is inhibited in caspase-3^{-/-} neurons, indicating the potential for RCAN1 to induce β -cell apoptosis via this enzyme (23). The large *in vitro* increase in ROS production under hyperglycemic conditions in RCAN1^{ox} islets illustrates that *in vivo* oxidative stress is likely higher in RCAN1^{ox} islets once RCAN1^{ox} mice become diabetic. Oxidative stress induces apoptosis in β -cells and reduces β -cell proliferation (24). Additionally, loss of calcineurin function reduces β -cell proliferation (10) and loss of *NeuroD* function, which is down-regulated in RCAN1^{ox} islets, reduces β -cell generation, and increases apoptosis (25). The loss of β -cells in RCAN1^{ox} mice may also be indirectly due to the effects of hyperglycemia itself. Chronic hyperglycemia has a clear link to increased β -cell apoptosis and reduced proliferation (26). Therefore, several mechanisms may explain how RCAN1 overexpression results in loss of β -cells.

Not only are β -cells lost as RCAN1^{ox} mice age but the secretory capacity of remaining RCAN1^{ox} β -cells is also reduced. The negative effect of RCAN1 on insulin release is not due to reduced insulin synthesis or altered localization of insulin granules or vesicle docking at the plasma membrane. The smaller size of the insulin granules in RCAN1^{ox} β -cell LDCVs and increase in the number of empty vesicles indicates problems with vesicle loading. The ZnT-8 vesicle zinc transporter controls insulin loading into vesicles and is inhibited by calcineurin inactivity and RCAN1 overexpression (27). Although this could explain the smaller insulin granule size and higher number of empty vesicles in RCAN1^{ox} β -cells, these effects are relatively minor.

Basal insulin release and first-phase GSIS are each reduced in RCAN1^{ox} β -cells, similar to secretory defects occurring in islets isolated from type 2 diabetes patients (28). RCAN1 may affect distal steps in the exocytosis pathway as occurs in RCAN1^{ox} adrenal chromaffin cells in which the number of exocytotic events and the amount released per vesicle are reduced (11). However, this would not explain the specific decrease in first-phase, but not second-phase, GSIS. K_{ATP} channel-dependent mechanisms drive first-phase GSIS (29), whereas K_{ATP} channels are required only as an initiating event in the sustained

second-phase insulin response (30). The reduced *Glut2* and glucokinase expression in RCAN1^{ox} islets may limit glucose 6-phosphate availability for oxidative phosphorylation and glucose-regulated K_{ATP} channel closure and potentially explain reduced first-phase GSIS. Whether the overexpression of RCAN1 affects oxidative phosphorylation and K_{ATP} channel function resulting in altered first-phase but normal second-phase insulin secretion is unresolved and will be a matter of future study.

RCAN1^{ox} β -cell mitochondria are smaller and produce more ROS *in vitro* when exposed to high glucose. Increased RCAN1 expression in *Drosophila* neurons promotes mitochondrial fission and ROS production (12), and hyperglycemia increases mitochondrial fission through increased expression of dynamin-related protein 1 (31). Under conditions of chronic high glucose or free fatty acid exposure, β -cell mitochondria lose their ability to undergo fusion that would otherwise prevent β -cell apoptosis under such conditions (32). From the present study, we cannot conclude whether elevated RCAN1 expression directly reduced mitochondrial size or whether the aberrantly high hyperglycemia-induced ROS levels present in RCAN1^{ox} β -cells is the cause. Our data raise the interesting possibility that RCAN1 may act as a link between hyperglycemia, mitochondrial fission, ROS production, and β -cell apoptosis. Greater glucose-stimulated ROS production in RCAN1^{ox} islets than in WT islets was also observed. Glucose normally enters the glycolytic pathway and preferentially undergoes oxidative phosphorylation in β -cells. Under hyperglycemic conditions, however, glucose is metabolized via alternative pathways (33). These pathways include glyceraldehyde autophosphorylation, PKC activation, dicarbonyl formation, sorbitol metabolism, and hexosamine metabolism, which produce more ROS than oxidative phosphorylation (34, 35). Changes in glucose metabolism via these pathways in RCAN1^{ox} β -cells might therefore underlie the increased ROS production we observed in RCAN1^{ox} islets.

The age of onset of fasting hyperglycemia, glucose intolerance, hypoinsulinemia, islet shrinkage, and the reduced expression of multiple key β -cell genes in RCAN1^{ox} mice is similar to changes reported in mice displaying a loss of β -cell calcineurin activity (10). This indicates that these changes in RCAN1^{ox} mice may be due to reduced calcineurin activity caused by higher RCAN1 expression. However, some of the observed differences in RCAN1^{ox} β -cells in mitochondrial size, ROS production, GSIS, and vesicle filling have not been previously associated directly with calcineurin activity. Elucidating the pathways underlying RCAN1 regulation of β -cell function and identifying those that are dependent and independent of effects on

calcineurin activity will be an important area of future research.

Loss of β -cells and ensuing hypoinsulinemia is the ultimate cause of both type 1 and type 2 diabetes. In type 1 diabetes, β -cell death is caused by sudden autoimmune destruction. We do not feel this mechanism underlies the diabetes in our RCAN1^{ox} mouse model because these mice lose β -cells after 40–60 d and gradually display worsening hyperglycemia. In type 2 diabetes, insulin resistance causes an increase in β -cell number and enables compensatory hyperinsulinemia to maintain glucose homeostasis in humans. However, unknown changes, which are associated with glucotoxicity-induced oxidative stress, lead to an increase in β -cell apoptosis, a relative inadequacy of insulin, poorly controlled blood glucose, and ultimately type 2 diabetes. The finding that chronic, but not acute, hyperglycemia induces RCAN1 expression raises the potential for RCAN1 to be involved in the progressive β -cell dysfunction occurring in type 2 diabetes. By contrast, in primary neuronal cells, the short-term induction of RCAN1 expression is thought to have a protective effect, whereas prolonged elevated expression has a detrimental effect on cell survival (36). Our *in vivo* data illustrate that a chronic increase in RCAN1 expression leads to β -cell loss, hypoinsulinemia, and diabetes. Although 6 d of high glucose in WT mice causes a 1.5-fold induction of RCAN1 expression, the effect of more prolonged hyperglycemia on the level of RCAN1 expression is unknown. Our RCAN1^{ox} mouse model has a 3.5- to 5-fold increase in islet RCAN1 expression yet develops hyperglycemia only at 60–70 d of age. We hypothesize that when hyperglycemia occurs over time, as occurs during the progression of both type 1 and type 2 diabetes, RCAN1 expression will increase, resulting in β -cell dysfunction, β -cell loss, and hypoinsulinemia. Our findings, therefore, not only link RCAN1 to the control of β -cell number, secretion, gene expression, and mitochondrial function but also identify that RCAN1 is regulated by chronic hyperglycemia, and as such, increased RCAN1 expression has the potential to underlie β -cell dysfunction and hypoinsulinemia in diabetes.

Acknowledgments

Address all correspondence and requests for reprints to: Dr. Damien Keating, Department of Human Physiology and Centre for Neuroscience, Flinders University, Adelaide, Bedford Park SA 5042, Australia. E-mail: damien.keating@flinders.edu.au.

Disclosure Summary: The authors have nothing to disclose.

This work was supported by a BioInnovation SA Fellowship, Australian Research Council Future Fellowship, National

Health and Medical Research Council Project Grant, and Diabetes Australia Research Trust Grant.

References

- Poitout V, Robertson RP 2002 Secondary β -cell failure in type 2 diabetes: a convergence of glucotoxicity and lipotoxicity. *Endocrinology* 143:339–342
- Ermak G, Morgan TE, Davies KJ 2001 Chronic overexpression of the calcineurin inhibitory gene DSCR1 (Adapt78) is associated with Alzheimer's disease. *J Biol Chem* 276:38787–38794
- Lin HY, Michtalik HJ, Zhang S, Andersen TT, Van Riper DA, Davies KK, Ermak G, Petti LM, Nachod S, Narayan AV, Bhatt N, Crawford DR 2003 Oxidative and calcium stress regulate DSCR1 (Adapt78/MCIP1) protein. *Free Radic Biol Med* 35:528–539
- Tang C, Han P, Oprea AI, Lee SC, Gyulhandanyan AV, Chan GN, Wheeler MB, Giacca A 2007 Evidence for a role of superoxide generation in glucose-induced β -cell dysfunction in vivo. *Diabetes* 56:2722–2731
- Fuentes JJ, Genescà L, Kingsbury TJ, Cunningham KW, Pérez-Riba M, Estivill X, de la Luna S 2000 DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. *Hum Mol Genet* 9:1681–1690
- Kingsbury TJ, Cunningham KW 2000 A conserved family of calcineurin regulators. *Genes Dev* 14:1595–1604
- Rothermel B, Vega RB, Yang J, Wu H, Bassel-Duby R, Williams RS 2000 A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. *J Biol Chem* 275:8719–8725
- Casas C, Martínez S, Pritchard MA, Fuentes JJ, Nadal M, Guimera J, Arbonés M, Flórez J, Soriano E, Estivill X, Alcántara S 2001 Dscr1, a novel endogenous inhibitor of calcineurin signaling, is expressed in the primitive ventricle of the heart and during neurogenesis. *Mech Dev* 101:289–292
- Ermak G, Harris CD, Davies KJ 2002 The DSCR1 (Adapt78) isoform 1 protein calcipressin 1 inhibits calcineurin and protects against acute calcium-mediated stress damage, including transient oxidative stress. *FASEB J* 16:814–824
- Heit JJ, Apelqvist AA, Gu X, Winslow MM, Neilson JR, Crabtree GR, Kim SK 2006 Calcineurin/NFAT signalling regulates pancreatic β -cell growth and function. *Nature* 443:345–349
- Keating DJ, Dubach D, Zanin MP, Yu Y, Martin K, Zhao YF, Chen C, Porta S, Arbonés ML, Mittaz L, Pritchard MA 2008 DSCR1/RCAN1 regulates vesicle exocytosis and fusion pore kinetics: implications for Down syndrome and Alzheimer's disease. *Hum Mol Genet* 17:1020–1030
- Chang KT, Min KT 2005 *Drosophila melanogaster* homolog of Down syndrome critical region 1 is critical for mitochondrial function. *Nat Neurosci* 8:1577–1585
- Baek KH, Zaslavsky A, Lynch RC, Britt C, Okada Y, Siarey RJ, Lensch MW, Park IH, Yoon SS, Minami T, Korenberg JR, Folkman J, Daley GQ, Aird WC, Galdzicki Z, Ryeom S 2009 Down's syndrome suppression of tumour growth and the role of the calcineurin inhibitor DSCR1. *Nature* 459:1126–1130
- Rothermel BA, McKinsey TA, Vega RB, Nicol RL, Mammen P, Yang J, Antos CL, Shelton JM, Bassel-Duby R, Olson EN, Williams RS 2001 Myocyte-enriched calcineurin-interacting protein, MCIP1, inhibits cardiac hypertrophy in vivo. *Proc Natl Acad Sci USA* 98:3328–3333
- Prentki M, Nolan CJ 2006 Islet β -cell failure in type 2 diabetes. *J Clin Invest* 116:1802–1812
- Barreto SG, Bazargan M, Zotti M, Hussey DJ, Sukocheva OA, Peiris H, Leong M, Keating DJ, Schlothe AC, Carati CJ, Smith C, Toouli J, Saccone GT 2011 Galanin receptor 3: a potential target for acute pancreatitis therapy. *Neurogastroenterol Motil* 23:e141–e151

17. Cantley J, Selman C, Shukla D, Abramov AY, Forstreuter F, Esteban MA, Claret M, Lingard SJ, Clements M, Harten SK, Asare-Anane H, Batterham RL, Herrera PL, Persaud SJ, Duchon MR, Maxwell PH, Withers DJ 2009 Deletion of the von Hippel-Lindau gene in pancreatic β -cells impairs glucose homeostasis in mice. *J Clin Invest* 119:125–135
18. Mythili MD, Vyas R, Akila G, Gunasekaran S 2004 Effect of streptozotocin on the ultrastructure of rat pancreatic islets. *Microsc Res Tech* 63:274–281
19. Gunton JE, Kulkarni RN, Yim S, Okada T, Hawthorne WJ, Tseng YH, Roberson RS, Ricordi C, O'Connell PJ, Gonzalez FJ, Kahn CR 2005 Loss of ARNT/HIF1 β mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell* 122:337–349
20. Makino A, Scott BT, Dillmann WH 2010 Mitochondrial fragmentation and superoxide anion production in coronary endothelial cells from a mouse model of type 1 diabetes. *Diabetologia* 53:1783–1794
21. Gurda GT, Crozier SJ, Ji B, Ernst SA, Logsdon CD, Rothermel BA, Williams JA 2010 Regulator of calcineurin 1 controls growth plasticity of adult pancreas. *Gastroenterology* 139:609–619, 619.e1–e6
22. Movsesyan VA, Yakovlev AG, Dabaghyan EA, Stoica BA, Faden AI 2002 Ceramide induces neuronal apoptosis through the caspase-9/caspase-3 pathway. *Biochem Biophys Res Commun* 299:201–207
23. Sun X, Wu Y, Chen B, Zhang Z, Zhou W, Tong Y, Yuan J, Xia K, Gronemeyer H, Flavell RA, Song W 2011 Regulator of calcineurin 1 (RCAN1) facilitates neuronal apoptosis through caspase-3 activation. *J Biol Chem* 286:9049–9062
24. Zhang Z, Liew CW, Handy DE, Zhang Y, Leopold JA, Hu J, Guo L, Kulkarni RN, Loscalzo J, Stanton RC 2010 High glucose inhibits glucose-6-phosphate dehydrogenase, leading to increased oxidative stress and β -cell apoptosis. *FASEB J* 24:1497–1505
25. Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ 1997 Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in β 2/neuroD-deficient mice. *Genes Dev* 11:2323–2334
26. Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY 2003 Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic β -cell turnover and function. *Diabetes* 52:726–733
27. Kim I, Kang ES, Yim YS, Ko SJ, Jeong SH, Rim JH, Kim YS, Ahn CW, Cha BS, Lee HC, Kim CH 2011 A low-risk Z η T-8 allele (W325) for post-transplantation diabetes mellitus is protective against cyclosporin A-induced impairment of insulin secretion. *Pharmacogenomics J* 11:191–198
28. Del Prato S, Marchetti P, Bonadonna RC 2002 Phasic insulin release and metabolic regulation in type 2 diabetes. *Diabetes* 51(Suppl 1):S109–S116
29. Cook DL, Hales CN 1984 Intracellular ATP directly blocks K⁺ channels in pancreatic B-cells. *Nature* 311:271–273
30. Henquin JC, Ravier MA, Nenquin M, Jonas JC, Gilon P 2003 Hierarchy of the β -cell signals controlling insulin secretion. *Eur J Clin Invest* 33:742–750
31. Edwards JL, Quattrini A, Lentz SI, Figueroa-Romero C, Cerri F, Backus C, Hong Y, Feldman EL 2010 Diabetes regulates mitochondrial biogenesis and fission in mouse neurons. *Diabetologia* 53:160–169
32. Molina AJ, Wikstrom JD, Stiles L, Las G, Mohamed H, Elorza A, Walzer G, Twig G, Katz S, Corkey BE, Shirihai OS 2009 Mitochondrial networking protects β -cells from nutrient-induced apoptosis. *Diabetes* 58:2303–2315
33. Robertson RP 2004 Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet β -cells in diabetes. *J Biol Chem* 279:42351–42354
34. Wolff SP, Dean RT 1987 Glucose autooxidation and protein modification. The potential role of 'autooxidative glycosylation' in diabetes. *Biochem J* 245:243–250
35. Brownlee M 2001 Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820
36. Porta S, Serra SA, Huch M, Valverde MA, Llorens F, Estivill X, Arbonés ML, Martí E 2007 RCAN1 (DSCR1) increases neuronal susceptibility to oxidative stress: a potential pathogenic process in neurodegeneration. *Hum Mol Genet* 16:1039–1050