Sorcin links pancreatic β cell lipotoxicity to ER Ca\(^{2+}\) stores.

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ABSTRACT

Preserving β cell function during the development of obesity and insulin resistance would limit the worldwide epidemic of type 2 diabetes (T2DM). Endoplasmic reticulum (ER) calcium (Ca\(^{2+}\)) depletion induced by saturated free fatty acids and cytokines causes β cell ER stress and apoptosis, but the molecular mechanisms behind these phenomena are still poorly understood. Here, we demonstrate that palmitate-induced sorcin (SRI) down-regulation, and subsequent increases in glucose-6-phosphatase catalytic subunit-2 (G6PC2) levels contribute to lipotoxicity. SRI is a calcium sensor protein involved in maintaining ER Ca\(^{2+}\) by inhibiting ryanodine receptor activity and playing a role in terminating Ca\(^{2+}\)-induced Ca\(^{2+}\) release. G6PC2, a GWAS gene associated with fasting blood glucose, is a negative regulator of glucose-stimulated insulin secretion (GSIS). High fat feeding in mice and chronic exposure of human islets to palmitate decreases endogenous SRI expression while levels of G6PC2 mRNA increase. Sorcin null mice are glucose intolerant, with markedly impaired GSIS and increased expression of G6pc2. Under high fat diet, mice overexpressing SRI in the β cell display improved glucose tolerance, fasting blood glucose and GSIS, whereas G6PC2 levels are decreased and cytosolic and ER Ca\(^{2+}\) are increased in transgenic islets. SRI may thus provide a target for intervention in T2DM.
INTRODUCTION

Pancreatic β cell dysfunction is central to the pathogenesis of Type 2 diabetes (T2DM). During the progression of obesity and insulin resistance, pancreatic islets of Langerhans initially increase β cell mass and overproduce insulin (1). The increase in biosynthetic demand induced by chronic hyperglycaemia activates the unfolded protein response (UPR), while increases in circulating free fatty acids and cytokines lower endoplasmic reticulum (ER) calcium (Ca\(^{2+}\)) stores (2, 3), triggering ER stress and apoptosis if prolonged (4). The molecular mechanisms linking lipotoxicity and associated inflammation (5) to ER Ca\(^{2+}\) stores are largely unknown. Sorcin (gene name SRI), is a 22 kDa member of the penta-EF-hand family of calcium binding proteins that undergoes Ca\(^{2+}\)-dependent conformational changes (6-10). Sorcin is highly conserved amongst mammals and strongly expressed in primary mouse islets (11). In extra-pancreatic cells, notably cardiac myocytes, sorcin associates with the ryanodine receptor (RyR) (12), the pore-forming α\(_1\) subunit of voltage-dependent L-type Ca\(^{2+}\) channels (L-type VDCC) (13), and with sarcoendoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) pumps (14), to modulate excitation-contraction coupling through changes in intracellular Ca\(^{2+}\) homeostasis (12). Sorcin inhibits RyR activity (15), and plays a role in terminating Ca\(^{2+}\)-induced Ca\(^{2+}\) release (12), an inherently self-sustaining mechanism which, if unchecked, may deplete intracellular Ca\(^{2+}\) stores (16).

We have recently shown that siRNA-mediated knock-down of sorcin in MIN6 insulinoma β cells resulted in an apparent reduction in ER Ca\(^{2+}\) stores as judged by stimulation with an inositol 1,4,5-triphosphate (IP3) mobilizing agonist, and an inhibition of glucose-stimulated insulin secretion (GSIS) (17). These data indicated that sorcin may be
required to maintain intracellular Ca\textsuperscript{2+} stores, possibly through its known capacity to inhibit RyRs (15) and activate SERCA pumps (14).

Since we and others have demonstrated that elevated palmitate (2) and cytokine levels (18, 19) cause ER stress and apoptosis in pancreatic β cells at least in part by decreasing ER Ca\textsuperscript{2+} stores (3), it follows that sorcin overexpression might protect against ER stress induced by inflammation and lipotoxicity. Indeed, recent data in human islets showed a decrease in sorcin expression induced by TNFα (20).

In the present report we test this hypothesis using (i) mice bearing null alleles of the sorcin gene (Sri\textsuperscript{-/-}), (ii) transgenic mouse lines overexpressing sorcin in the pancreatic β cell, and (iii) adenovirus-mediated overexpression of sorcin in isolated human and murine islets.

**RESEARCH DESIGN AND METHODS**

*Generation of transgenic mice overexpressing sorcin in pancreatic β cells.* Murine sorcin cDNA (17) was inserted in pBI-L vector (PvuII-NotI sites), which contains a bidirectional Tet-responsive promoter driving the expression of both mSRI and firefly luciferase (21). After injection into the pronucleus of 0.5 day old pure C57BL/6 fertilised oocytes (Embryonic Stem Cell and Transgenic Facility, MRC, London, UK) two founders, bearing one and 10 copies of the transgene (TetOn-Sri-1 and TetOn-Sri-10), were identified by PCR screening. β cell selectivity was achieved using the Tet-on system and RIP7-rtTA mice, which express the reverse tetracycline transactivator (rtTA) under the control of the rat insulin 2 promoter (21). Hemizigous TetOn-Sri-1 or -10 mice were crossed with homozygous RIP7-rtTA mice to generate double hemizigous TetOn-Sri/RIP7-rtTA (hereafter named SRI-tg1 and SRI-tg10) and single hemizigous RIP7-rtTA as littermate controls. Doxycycline (0.5
g/L in drinking water) and a high fat diet (HFD, 60% Kcal as fat, mainly saturated) were administered from 4 weeks of age unless specified otherwise.

**Generation of sorcin knockout mice.** Homozygous sorcin null mice (Sri^-/-) on a 129S1/SvImJ genetic background were generated by homologous recombination as described (22). Briefly, the targeting construct was generated by flanking exon 3, present in both sorcin isoforms (Accession: NM_001080974.2 and NM_025618.3) with loxP sites for the Cre recombinase and inserting a phosphoglycerol kinase promoter-driven neomycin selection cassette flanked by an additional loxP site in the intron between exons 3 and 4.

**Intraperitoneal glucose and insulin tolerance tests.** Mice were fasted overnight for 14 h. Glucose solution (20% D-glucose/water, w/v, 1-3g/kg body weight) or human regular insulin solution (Sigma #19278, 0.5 or 1 U/kg) were administrated intraperitoneally and blood glucose was measured from the tail vein at 0, 15, 30, 60, 90 and 120 min using an ACCU-CHECK AVIVA glucometer (Roche). Plasma insulin levels were measured using an ultrasensitive mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL), and plasma glucose was assessed by Glucose Assay Kit (Abcam #65333) when above the glucometer detection limit.

**Plasmids and adenoviral vectors.** Plasmid pGL3-hG6PC2(-1075+124), containing the proximal promoter of the human G6PC2 gene upstream of luciferase reporter gene, was generated by PCR using human genomic DNA and the following primers: Forward_5'-
ACACGGTACCACCTAGACACAATCCAGCTCTCTCT and Reverse_5’-ACACAAGCTTTAAATGAAAAAGATATTCCCTGGGG. The resulting 1220-bp fragment was subcloned into pCR2.1 by TA cloning, digested by KpnI–XhoI and subcloned into pGL3basic. A nuclear factor of activated T-cells (NFAT) luciferase reporter containing three tandem repeats of a 30-bp fragment of the IL-2 promoter for analysis of NFAT activity was a gift from Dr Toren Finkel (23). p5xATF6-GL3, containing five tandem repeats of ATF6 binding sites, was a gift from Ron Prywes (24). Plasmids pLKO.1-shSc (scrambled), pLKO.1-shSRI144, and pLKO.1-shSRI457 were constructed using the pLKO.1-TRC cloning vector from Addgene (plasmid #10878, protocol http://www.addgene.org/tools/protocols/plko/) and the oligonucleotides presented in Suppl. Table 1. Plasmid pAd-hSRI was generated subcloning the human sorcin cDNA (pDNR-LIB-SRI) in pAdTrackCMV (BglIII-HindIII sites). All the constructs were verified by DNA sequencing. The adenovirus Ad-hSRI-GFP was subsequently produced as in (17). Ad-mSRI-GFP encoding the murine sorcin cDNA and Ad-Null-GFP (empty vector) were described in (17).

Cell culture, transfection and luciferase assays. MIN6 β-cells were used between passages #24 and #39 as in (17). Human and rat β-cell lines 1.1B4 and INS1(832/13), and HEK293 cells were cultured as in (25). Cells were transfected using Lipofectamine 2000 and Opti-Mem (Invitrogen) and luciferase assays were performed using a Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions.

Human and mouse islets of Langerhans isolation and culture. Human islets from normoglycaemic donors were cultured as in (26). Donor characteristics are presented in
Suppl. Table 2. Pancreatic mouse islets were isolated and cultured as previously described (27). Transgenic islets were cultured in the presence of 0.5 µg/ml doxycycline to sustain transgene expression. Human and wild type mouse islets (10-week-old C57BL/6 mice) were transduced with Ad-hSRI-GFP, Ad-mSRI-GFP or Ad-Null-GFP adenoviruses, at a multiplicity of infection of 100 for 48 h prior to total RNA extraction, GSIS or intracellular Ca\(^{2+}\) imaging.

**RNA extraction, cDNA synthesis and quantitative PCR analysis.** Total RNA was extracted, reversed transcribed and analysed as described in (28). Primers for SYBR Green assays are presented in Suppl. Table 3. Expression of each gene was normalised to β-actin and fold change in mRNA expression versus controls calculated using the 2\(^{-\Delta\Delta CT}\) method.

**Microarray analysis.** Total RNA isolated from islets from 8-week-old, HFD-fed six SRI-tg10 male mice and six RIP7rtTA (littermate controls) was sent to the High Throughput Genomics Facility, Wellcome Trust Centre for Human Genetics, Oxford University, U.K., and analysed on Illumina MouseWG6-v2 Expression BeadChips.

**Ex vivo glucose-stimulated insulin secretion.** Insulin secretion assays on murine and human islets were performed as described in (29). Secreted and total insulin content were quantified using HTRF Insulin kit (Cisbio).

**Palmitate treatment.** Human islets were cultured for 72 h with either BSA or 0.5 mM BSA-palmitate in 5.5 mM glucose RPMI and MIN6 cells were transduced with Ad-mSRI-GFP or
Ad-Null-GFP adenoviruses for 24 h, prior 48 h treatment with BSA or 0.5 mM BSA-palmitate in 25 mM glucose DMEM before total RNA extraction and qRT-PCR analysis.

**Protein extraction and Western blotting.** Western blotting were performed as in (29) using mouse monoclonal anti-sorcin (Invitrogen 25B3; 1:300) and mouse monoclonal anti-alpha tubulin (Sigma #T5168; 1:10,000-20,000) antibodies.

**Cytosolic calcium imaging.** Imaging of free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)) in isolated islets was performed using the trappable intracellular fluorescent Ca\(^{2+}\) dye Fura-2\(^{TM}\)-AM (Invitrogen) (27). Imaging data were analysed with ImageJ software using an in-house macro and the fluorescence emission ratios were derived after subtracting background fluorescence.

**Endoplasmic reticulum calcium imaging.** Clusters of isolated islets were transduced for 48 h with an adenovirus encoding the low Ca\(^{2+}\)-affinity sensor D4 addressed to the ER, Ad-RIP-D4ER, as in (30). Prior to acquisitions, cells were preincubated in Kreb’s Ringer bicarbonate-HEPES (KRBH) media (17) containing 3 mM glucose for 1 h at 37°C and were perfused with KRBH supplemented with 17 mM glucose and 250 µM diazoxide (Sigma #D9035) with the subsequent consecutive additions of 100 µM acetyl-choline (Sigma #A2661) and 1 µM thapsigargin (Calbiochem #586005). Image analysis was performed as above.

**Immunohistochemistry and immunofluorescence quantification of β and α cell mass.** Analysis were performed as described in (28).
**Statistical analysis.** Data are presented as means ± standard error (SEM). Significance was assessed by appropriate unpaired or paired two-tailed Student’s t-tests, or one- or two-way ANOVA as indicated, using GraphPad Prism 6.0 or Microsoft Excel. P<0.05 was considered significant.

**Study approval.** Studies involving human islets were approved by the National Research Ethics Committee London as detailed in (26). All procedures involving animals received ethical approval and were compliant with U.K. Animals Scientific Procedures Act, 1986 or approved by the University Committee on Use and Care of Animals (University of Michigan, US). Animals were housed 2-5 per individually-ventilated cage in a pathogen-free facility with a 12-h light-dark cycle and had free access to food and water.

**RESULTS**

**Sorcin is necessary for normal glucose tolerance and protects against lipotoxicity in vivo.**

We previously reported that sorcin silencing in MIN6 cells leads to a complete abolition of ATP-evoked Ca\(^{2+}\) release from intracellular stores and an inhibition of GSIS (17). These findings prompted us to investigate the roles of sorcin in β cell pathophysiology provoked by lipotoxicity, a condition known to trigger ER stress and β cell failure (2).

In line with our findings in cell lines (17), sorcin null mice (Sri\(^{-/-}\), standard chow-fed) displayed decreased glucose tolerance compared to sex-, weight- and age-matched wild type (WT) controls during intraperitoneal glucose tolerance tests (IPGTT, AUC, arbitrary units,
WT vs Sri–/–, 2-month old: 43.5 ± 1.64 vs 48.0 ± 1.1, n= 6-10, p<0.05; 9-month old: 39.2 ± 2.5 vs 49.1 ± 1.9, n= 4-7, p<0.01, Fig. 1A,B).

To determine whether sorcin overexpression might be protective against β cell stress, we generated transgenic mice overexpressing SRI in the pancreatic β cells on the C57BL/6 genetic background, since males of this strain become glucose intolerant and insulin resistant under a HFD (31). Sorcin mRNA and protein levels were increased by at least 2-fold in isolated islets from SRI-tg1 and SRI-tg10 mice compared to those from littermate controls (Suppl. Fig. 1).

Glucose tolerance was improved in HFD-fed SRI-tg1 and SRI-tg10 male mice compared to their littermate controls during IPGTTs (AUC, arbitrary units, controls vs SRI-tg1: 128.7 ± 6.1 vs 101.2 ± 8.1, n=7-8, p<0.05; controls vs SRI-tg10: 95.8 ± 5.4 vs 73.0 ± 2.4, n=9-13, p<0.001; Fig 1C, D), despite similar insulin sensitivity as assessed by intraperitoneal insulin tolerance tests (IPITT, Fig. 1E, F, left panels) and body weights (Fig. 1E, F, right panels). Remarkably, the above phenotype was not apparent in vivo in the absence of β cell stress i.e. during normal chow feeding (Suppl. Fig. 2).

Sorcin enhances glucose-stimulated insulin secretion without increasing pancreatic β cell mass. We next investigated whether the changes in glucose tolerance observed in SRI-tg and SRI–/– mice were secondary to changes in insulin secretion. In vivo glucose-stimulated insulin release was assessed in SRI-tg10 and SRI–/– mice by IPGTTs (3g glucose/kg body weight). As shown in Fig. 2A (top panel), plasma insulin concentrations were significantly higher at 30 min in SRI-tg10 compared to controls (plasma insulin, ng/ml, SRI-tg10 vs controls, 30 min: 0.60 ± 0.06 vs 0.43 ± 0.05, p<0.05, n=5-7), despite similar concomitant blood glucose values
Conversely, $Srt^{-/-}$ males showed a marked impairment of GSIS compared to WT controls, with plasma insulin concentrations barely rising during IPGTT (AUC insulin, WT vs $Srt^{-/-}$, arbitrary units: $1.01 \pm 0.23 \ vs \ 0.53 \pm 0.10$, $n=4-6$, $p<0.05$, Fig. 2B, top panel), despite robust increases in associated blood glucose values (Fig. 2B bottom panel).

We next explored whether the enhanced GSIS observed in SRI-tg10 islets might be secondary to an increase in β cell mass. As shown in Figure 2C-E, there were no significant changes in mean pancreas surface, islet size, individual β or α cell mass, although there was a significant decrease in β to α cell ratio.

The sorcin-induced improvement in in vivo GSIS was also observed ex vivo in islets isolated from SRI-tg1/10 mice and from human and mouse islets transduced for 48h with an adenovirus encoding sorcin. Indeed, when stimulated with 17 mM glucose, SRI-tg10 islets secreted 55% more insulin compared to control islets (insulin, % of total, controls vs SRI-tg10: $0.372 \pm 0.02 \ vs \ 0.577 \pm 0.07$, $n=3$, $p<0.05$), without any changes in insulin secretion at 3 mM glucose (Fig. 2F), whereas SRI-tg1 islets secreted 68% more insulin at high glucose than controls (Suppl. Fig. 3). Adenovirus-mediated overexpression of sorcin consistently increased GSIS in human (Fig. 2G) and mouse (Fig. 2H) islets at 17 mM glucose compared to islets transduced with a null-GFP virus.

**Sorcin improves cytosolic $Ca^{2+}$ fluxes and increases ER $Ca^{2+}$ stores.** We next assessed whether the enhanced GSIS observed following sorcin overexpression was associated with changes in intracellular $Ca^{2+}$ dynamics. Islets isolated from HFD-fed SRI-tg1 and SRI-tg10 mice were loaded with Fura-2, and perifused sequentially with low (3mM) and elevated (17mM) glucose concentrations. High glucose elicited a greater $[Ca^{2+}]_{cyt}$ response in sorcin-
overexpressing islets compared to controls (AUC, arbitrary units, controls vs SRI-tg1: 100.0 ± 5.0 vs 120.1 ± 5.0, n=3, p<0.05, Fig. 3A; controls vs SRI-tg10: 100.0 ± 17.80 vs 149.9 ± 16.2, n=3, p<0.05, Fig. 3B). Likewise, transduction of dissociated human (Suppl. Fig. 4) or mouse (not shown) islets in vitro with sorcin-encoding adenoviruses significantly increased the number of islets displaying strong and high-amplitude [Ca^{2+}]_cyt oscillations in response to 17 mM glucose.

Free Ca^{2+} in the ER ([Ca^{2+}]_{ER}) was measured in clusters of isolated islets from HFD-fed SRI-tg1, SRI-tg10 and their littermate controls, transduced for 48 h with an adenovirus encoding the low Ca^{2+}-affinity sensor D4 addressed to the ER under the control of the insulin promoter, Ad-RIP-D4ER (30), and incubated in 17mM glucose with the addition of 250 µM diazoxide to fully open ATP-sensitive K^+ channels and prevent extracellular Ca^{2+} influx (30). Following acetylcholine-induced ER Ca^{2+} release, transgenic islets experienced a larger fall in [Ca^{2+}]_{ER} than control islets, indicating of a higher initial [Ca^{2+}]_{ER} content (Fig. 3C-D). We next fully depleted the ER Ca^{2+} stores in islets isolated from HFD-fed SRI-tg10 male mice and littermate controls using the SERCA pump inhibitor cyclopiazonic acid (CPA) (32, 33) before measuring [Ca^{2+}]_cyt in response to 17 mM glucose. Under these conditions, the subsequent increase in [Ca^{2+}]_cyt induced by high glucose was no longer significantly different between sorcin overexpressing islets and control islets (AUC [Ca^{2+}]_cyt, arbitrary units, controls vs SRI-tg10: 100.0 ± 13.5 vs 104.6 ± 10.3, n=3, NS, Suppl. Fig. 5). Taken together these results are consistent with a positive role for sorcin in GSIS and intracellular Ca^{2+} homeostasis, corroborating our in vitro data in MIN6 insulinoma cells (17).
**Sorcin regulates G6PC2 expression levels and reduces fasting blood glucose.** To further explore the underlying mechanisms behind sorcin’s actions, we performed a transcriptomic analysis of islets from HFD-fed SRI-tg10 mice and controls using oligonucleotide microarrays (GEO accession no.GSE72719; Ingenuity Pathway Analysis presented in Suppl. Table 4). Interestingly, G6pc2, one of the most highly expressed genes in β cells (9), was strongly repressed in islets from SRI-tg10 mice. Subsequent qRT-PCR analysis in isolated islets from SRI-tg10, SRI-tg1 and Srt<sup>−/−</sup> mice confirmed the inverse relationship between Sri and G6pc2 expression levels (Fig. 4A-F). Indeed, islets from SRI-tg1 and SRI-tg10 mice displayed 35% and 56% decreases in G6pc2 mRNA levels (Fig. 4D,E) while Sri mRNA levels were increased 20- and 42-fold, respectively (Fig. 4A,B). In islets from Srt<sup>−/−</sup> mice, sorcin expression was reduced by > 90% and there was a 3.6-fold increase in G6pc2 expression (Fig. 4C,F). G6PC2 is an islet-specific isoform of glucose-6-phosphatase which negatively regulates basal GSIS (9). G6PC2 is also a major determinant of fasting blood glucose in humans as revealed by genome-wide association studies (GWAS) (34). Accordingly, HFD-fed SRI-tg10 mice displayed lower fasting blood glucose throughout the study compared to controls (Fig. 4G).

**Lipotoxic conditions decrease endogenous sorcin expression in mouse and human islets while increasing G6PC2 and ER stress markers.** We next overexpressed sorcin in human islets with an adenoviral vector and likewise observed a reduction in G6PC2 mRNA levels, and in the levels of mRNA encoding the ER stress markers C/EBP homologous protein (CHOP) and glucose regulated protein 78/binding immunoglobulin protein (GRP78/BiP;Fig. 5A).
Given the apparent protection conferred by sorcin overexpression under lipotoxic conditions, we next investigated the regulation of endogenous sorcin during HFD in vivo and in human islets and MIN6 cells cultured in the presence of palmitate. Sorcin expression was down-regulated in islets from HFD-fed C57BL/6 (Fig. 5B) and DBA2J mice (Suppl. Fig. 6) compared to chow-fed mice. Furthermore, human islets incubated for 72h in the presence of palmitate and 5.5 mM glucose showed a significant reduction in sorcin expression and an increase in G6PC2 and CHOP expression (Fig. 5C). Similarly, lipotoxicity experiments in MIN6 cells also demonstrated a profound suppression of sorcin expression accompanied by a robust increase in G6pc2, Chop and Grp78/BiP mRNA after exposure to palmitate (Fig. 5D). Remarkably, adenovirus-mediated overexpression of sorcin during lipotoxic conditions in MIN6 cells prevented the increase in G6pc2, Chop and Grp78/BiP expression (Fig. 5E).

**Sorcin represses G6PC2 promoter activity through NFAT activation.** We next determined whether the repressive effect of sorcin on G6PC2 expression was transcriptionally mediated. As shown in Fig. 6A (DMSO, grey bar), overexpressed sorcin repressed the activity of a 1.2 kb proximal fragment of the human G6PC2 promoter (hG6PC2p) in MIN6 cells. Conversely, the activity of a reporter containing three tandem repeats of an NFAT binding site (3xNFATr) was significantly stimulated by sorcin (Fig. 6B, DMSO, grey bar), consistent with the increase in cytosolic [Ca$^{2+}$] induced by sorcin (35). In order to confirm the roles of NFAT and [Ca$^{2+}$]$_{cyt}$ in mediating the inhibitory effect of sorcin on G6PC2 expression, we showed that NFAT cDNA co-transfection repressed hG6PC2p activity while robustly stimulating 3xNFATr (Fig. 6A,B, DMSO, black bars). *In silico* Transfac® analysis revealed three putative NFAT binding sites on G6PC2 promoter (not shown). We next added diazoxide (Diaz, 100 µM, inhibiting Ca$^{2+}$ influx) and cyclosporine A (CsA, 0.2 µM, inhibiting NFAT
nuclear translocation) in the culture medium. As expected, both agents prevented the inhibitory and stimulatory effects of sorcin on hG6PC2p and 3xNFATr, respectively (Fig. 6A,B, Diaz & CsA, grey bars). Moreover, the addition of CsA stimulated the activity of hG6PC2p compared to DMSO, while Diaz inhibited it (Suppl. Fig. 7), confirming the repressive contribution of the NFAT signalling pathway. The suppressive effect of diazoxide, however, implies additional \([\text{Ca}^{2+}]_{\text{cyt}}\)-dependent stimulatory pathways. Intriguingly, the addition of Diaz and CsA (up 1 \(\mu\)M, not shown) did not prevent the inhibitory effects of NFAT on hG6PC2p but prevented the stimulatory effect of NFAT on 3xNFATr (Fig. 6A,B, Diaz & CsA, black bars).

**Sorcin activates ATF6 transcriptional activity.** The inverse relationship between sorcin expression and the ER stress markers CHOP and GRP78/BiP prompted us to investigate the effect of sorcin on the UPR. To study the ATF6 branch (36), we chose a reporter assay containing five tandem repeats of ATF6 binding sites (24), since ATF6 is cleaved in the Golgi in response to ER stress before translocating to the nucleus (37). The activity of the ATF6 luciferase reporter was reproducibly stimulated by tunicamycin and thapsigargin, two ER stress-inducers, and inhibited by the chemical chaperone, 4-PBA, confirming its sensitivity to ER stress (Fig. 7A-D). Sorcin co-transfection increased the activity of the ATF6 luciferase reporter in three \(\beta\) cell lines, i.e. MIN6, 1.1B4, and INS1 (832/13), as well as HEK293 cells, in basal (DMSO) and stimulated (thapsigargin and tunicamycin) conditions, but not in the presence of 4-PBA, compared to co-transfection with GFP (Fig. 7A-D). Conversely, sorcin silencing in MIN6 cells by shRNA decreased the activity of the ATF6 reporter (Fig. 7E-F). Sorcin overexpression did not affect XBP-1 splicing, used as a surrogate of the IRE-1 branch, either in basal conditions or after thapsigargin treatment of MIN6 (Fig.
DISCUSSION

The findings herein indicate that sorcin lies on a pathway linking β cell lipotoxicity to ER calcium and ER stress, representing a mechanism for dysregulation of β cell function under conditions of metabolic stress (Scheme Fig. 8). Thus, we show that sorcin is down-regulated in pancreatic β cells under conditions of lipotoxic stress (Fig. 5), whilst overexpression of sorcin is sufficient to protect against β cell failure and glucose intolerance during HFD (Fig. 1).

Interestingly, in the absence of β cell stress, i.e. during normal chow feeding, the role of sorcin in pancreatic β cells was less prominent. Nonetheless, forced sorcin expression enhanced GSIS and [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations in human islets from normoglycaemic donors as well as in islets from young chow-fed mice (Fig. 2 and Suppl. Fig. 4). Importantly, the observed increase in GSIS in our transgenic models was not due to an increase in β cell mass. Rather, we observed a decrease in the β to α cell ratio (Fig. 2), suggesting that the sorcin-overexpressing islets display enhanced function, and a possible resistance to HFD-induced hyperplasia (38). The improved GSIS observed in our HFD-fed SRI-tg1/10 mice is most likely secondary to the increases in glucose-induced intracellular Ca\(^{2+}\) fluxes (Fig. 3 and Suppl. Fig. 4). Although not tested here, the combined effects of RyR inhibition and SERCA activation by sorcin described in cardiomyocytes (14, 15) might thus explain the increased capacity of ER Ca\(^{2+}\) stores in SRI-tg1/10 islets (Fig. 3). In the rat heart, sorcin overexpression is associated with an increase in Ca\(^{2+}\) transients and enhanced cardiac contractility, rescuing diabetic contractile dysfunction (39). In β cells, a role for RyR, in particular RyR2, has
recently been supported by studies using “leaky” mutants, both in humans in mice, which
display glucose intolerance, decreased insulin secretion and islet ER stress (40, 41).

One intriguing finding of the present study was the inverse relationship between
sorcin and G6PC2 expression in islets and β cells. G6PC2 acts by hydrolysing glucose-6-
phosphate in the ER, thus opposing the action of the glucokinae (9, 42). Islets from G6pc2−/−
mice display increased cytosolic [Ca2+] and enhanced GSIS (9). This suggests that an
important mechanism of action of sorcin is to regulate G6PC2 expression to influence
calcium homeostasis, GSIS and ER stress. Interestingly, others have found that glucose
cycling and G6Pase activity were markedly enhanced in pancreatic islets of HFD-fed obese
hyperglycaemic mice, impairing GSIS (43). However, it is possible that G6PC2 also exerts
effects beyond glucose cycling and glycolytic flux (44) and earlier studies have linked
G6pase activity and G6P levels to cytosolic and ER Ca2+ concentrations, both in the liver and
in pancreatic β cells (45, 46).

Our study highlights several beneficial effects of sorcin in the β cell. For example,
sorcin-induced increase in intracellular Ca2+ activates NFAT signalling, which is fundamental
for maintaining the islet β cell phenotype (47), and whose inhibition is responsible for post-
transplantation diabetes caused by calcineurin inhibitors (48). Furthermore, sorcin
overexpression under lipotoxic conditions prevented the induction of the ER stress markers
CHOP and GRP78/BiP. Interestingly, ATF6 signalling was stimulated by sorcin, a change
which, unlike the other two branches of the UPR, i.e. PERK and IRE-1, is not usually
associated with apoptosis but with favourable outcomes (36, 49). We note that our data may
also have direct relevance for beta cell failure in humans. Thus, analysis of unpublished
results from the IMIDIA consortium (Solimena et al, manuscript in preparation) from large
sets of human donor islets indicates a significant positive correlation between SRI mRNA
levels and GSIS in both diabetic and non-diabetic islets, and a tendency towards lower sorcin levels in T2D patient-derived versus healthy islets.

Thus, agents which increase sorcin expression or activity may increase insulin secretion while protecting against β cell exhaustion.

AUTHOR CONTRIBUTIONS

AM: designing research studies, conducting experiments, acquiring data, analysing data and writing the manuscript; JP: conducting experiments, acquiring data and analysing data; XC: conducting experiments and acquiring data; LC: conducting experiments, acquiring data and writing the manuscript; JH: conducting experiments and acquiring data; NM: conducting experiments and acquiring data; PM, LP, DB, PJ and JAMS: provision of human islets; MI: bioinformatics analysis of IMIDIA data; CM, CGC, BT: provision of IMIDIA data; HHV: provision of Srt−/− mice and polyclonal anti-SRI antibody; GAR: designing research studies, analysing data, providing reagents, and writing the manuscript; IL: designing research studies, conducting experiments, acquiring data, analysing data, providing reagents, and writing the manuscript. IL is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors have declared that no conflict of interest exists.

ACKNOWLEDGEMENTS.

We are grateful to Diabetes UK for their financial support to IL (BDA:12/0004535), to the European Federation for the Study of Diabetes (EFSD) for the attribution of an Albert
Renold Travel Fellowships for Young Scientists to AM, and to the National Institutes of Health (NIH) for grants R01-HL120108 and R01-HL055438 to HHV. GAR was supported by grants from the Wellcome Trust (WT098424AIA), the MRC (Programme MR/J0003042/1), and BBSRC (BB/J015873/1). GAR is a Royal Society Wolfson Research Merit Award holder. The work leading to this publication also received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement nº 155005 (IMIDIA) (GAR, BT, CM, PM), resources of which are composed of a financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies’ in kind contribution.

We thank Anke Schulte (Sanofi Aventis, Frankfurt, Germany) and Michel Solimena (Technical University, Dresden, Germany) for sharing expression data on sorcin in human islets ahead of publication. We thank Pauline Chabosseau (Imperial College London) for help in designing macros for Ca\textsuperscript{2+} imaging and β cell mass measurements. We thank the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics (University of Oxford) for the generation of the Gene Expression data.

REFERENCES


FIGURE LEGENDS

Figure 1. Sorcin deletion impairs glucose tolerance while sorcin overexpression in β cells improves glucose tolerance during high fat diet. (A, B) IPGTT, (1 g glucose/kg) were performed in SD-fed (A) 2-month-old and (B) 9-month-old Sr↓/- male mice and sex-, weight- and aged-matched WT controls (n=4-10). (C, D) IPGTT, (1 g glucose/kg) were performed in HFD-fed (C) SRI-tg1 (n=8-9, 16-week-old) and (D) SRI-tg10 (n=9-11, 8-week-old) male mice and littermate controls. Right panels represent AUC of blood glucose concentration during IPGTTs. (E, F) IPITT were performed in HFD-fed (E) SRI-tg1 (n=8-9, 17-week-old, 1 U insulin /kg) and (F) SRI-tg10 (n=9-11, 9-week-old, 0.5 U insulin /kg) male mice and littermate controls. Right panels represent body weights for each group at the time of IPITTs. Values are means ± SEM. *p<0.05, **p<0.01, ***p<0.001; Two-way ANOVA.

Figure 2. Sorcin overexpression enhances GSIS without expansion of β cell mass whereas sorcin deletion impairs GSIS. (A-B) Plasma insulin concentration during 3 g glucose/kg IPGTTs were assessed in (A) HFD-fed SRI-tg10 male mice (n=5-7, 11-week-old) and (B) SD-fed Sr↓/- male mice (n=4-6, 9 months old) and their respective controls. Top left panels represent plasma insulin values whereas bottom panels represent corresponding blood glucose measurements. Top right panels represent AUC of plasma insulin concentrations. (C-F) Pancreatic β cell mass was evaluated in HFD-fed SRI-10 mice. Five consecutive pancreatic sections from 9-week-old SRI-tg10 and littermate control mice (n= 3-4) were immunostained for (C) insulin, glucagon and DAPI (scale bars = 50 µm) to quantify for (D) mean pancreas and islet size, (E) individual β cell and α cell area and β: α cell ratio as described in Methods. (F-H) Ex vivo insulin secretion assays were performed in response to 3
or 17 mM glucose (G3; G17) on isolated islets from (F) HFD-fed SRI-tg10 male mice (n=3, 27-week-old), (G) human cadaveric donors (n=3, see Supplemental Table 2 for donors characteristics) and (H) SD-fed WT C57BL/6 mice (n=4-5, 10-week-old) transduced with an adenovirus encoding sorcin-GFP or GFP only as indicated. *p<0.05; in vivo GSIS/IPGTT: Two-way ANOVA; β cell mass and ex vivo GSIS: Two-tailed Student’s t-tests.

Figure 3. Sorcin overexpression increases intracellular Ca^{2+} fluxes and ER Ca^{2+} stores. (A, B) Cytosolic Ca^{2+} levels ([Ca^{2+}]_{c}) were measured in isolated and dissociated islets loaded with Fura-2 from HFD-fed (A) SRI-tg1 (n= 3-4, 14-week-old) and (B) SRI-tg10 (n=3, 9-week-old) male mice incubated sequentially with low (3mM, G3) and high (17mM, G17) glucose concentrations as indicated. Right panels represent AUC of [Ca^{2+}]_{c}. (C, D) Acetylcholine (Ach)-induced ER Ca^{2+} release was measured in clusters of dissociated islets from HFD-fed (C) SRI-tg1 (n=4, 14-week-old) and (D) SRI-tg10 (n=3, 9-week-old) male mice transduced with Ad-RIP-D4ER adenovirus to measure [Ca^{2+}]_{ER} as stated in Methods. The islets were incubated in 17 mM glucose in the presence of diazoxide (Dz; 250 µM) to prevent extracellular Ca^{2+} influx. Left panels: representative calcium traces; right panels: quantification of the amplitude (∆) of ER Ca^{2+} depletion following treatment with Ach. *p<0.05, **p<0.01, ***p<0.001; [Ca^{2+}]_{c}: Two-way ANOVA; [Ca^{2+}]_{ER} ∆ : Two-tailed Student’s t-tests.

Figure 4. Sorcin regulates G6pc2 expression and decreases fasting blood glucose in vivo. Quantitative RT-PCR analysis of (A- C) Sri and (D- F) G6pc2 expression were performed in isolated islets from HFD-fed (A, D) SRI-tg1 male (n=3 mice/genotype, 27-week-old) (B, E) SRI-tg10 male (n=5 mice/genotype, 8-week-old), and (C, F) SD-fed SRI^{−/−} male (n=4-7
mice/genotype, 9 months old) mice and their respective controls. (G) Fasting blood glucose levels were measured in HFD-fed SRI-tg10 male and their littermate controls mice aged 8, 16 and 24 weeks as indicated (n= 9-11 mice per group, HFD from 4-week-old). *p<0.05, **p<0.01, ***p<0.001, Two-tailed Student’s t-tests.

Figure 5. Lipotoxicity decreases endogenous sorcin expression whereas sorcin overexpression prevents palmitate-induced G6PC2 and ER stress markers induction. (A) Quantitative RT-PCR analysis of SRI, G6PC2, CHOP and GRP78/BiP expression, were performed on human islets (n= 3 donors) transduced 48h with either sorcin (GFP-SRI) or null (GFP) adenoviruses as indicated. (B) Quantitative RT-PCR analysis of SRI expression were performed in isolated islets from chow or HFD-fed WT mice (n=4-5, 19-week-old females, HFD from 5-week-old). (C- E) Quantitative RT-PCR analysis of SRI, G6PC2, CHOP and/or GRP78/BiP expression, were performed on (C) human islets (n= 3 donors) treated 72h with 0.5 mM BSA-conjugated palmitate or BSA only, or on (D, E) MIN6 β cells transduced 24h with an adenovirus encoding GFP or sorcin-GFP followed by 48h treatment with 0.5 mM BSA-conjugated palmitate or BSA only. Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, Two-tailed Student’s t-tests.

Figure 6. Sorcin inhibits G6PC2 promoter activity. Promoter luciferase reporter studies were performed in MIN6 β cells co-transfected with either GFP (control), SRI or NFAT-GFP cDNAs and (A) -1075+124hG6PC2-Luci or (B) 3 tandem repeats of NFAT binding sites (3X NFAT-Luci) and pRL-CMV and treated with DMSO (0.1%), diazoxide (Diaz, 100 µM) or cyclosporin A (CsA, 0.2 µM) for a further 24h before cell lysis as indicated(n=3-4
independent experiments). Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, Two-tailed Student’s t-tests.

**Figure 7. Sorcin activates ATF6 transcriptional activity (A- D)** Promoter luciferase reporter studies were performed in clonal β cell lines (A) MIN6, (B) 1.1B4, (C) INS1(832/13), and (D) human embryonic kidney HEK cells. Cells were co-transfected with an artificial promoter containing five ATF6 binding sites (p5xATF6-GL3), pRL-CMV and either GFP or SRI cDNA for 24h and treated with DMSO (1%), 4-PBA (10mM), tunicamycin (10 mg/ml) or thapsigargin (10 mM) for a further 16-20h before cell lysis and luciferase assay. *ap<0.005 for the effect of treatment vs DMSO; bp<0.05 for the of SRI overexpression, unpaired two-tails Student’s t-tests. (E) Western blot showing the efficiency of two different sorcin shRNA constructs (shSRI-144 and shSRI-457) after 3 and 5 days in culture in MIN6 cells. Cells were transfected with either pLKO.1-shScrambled, -shSRI144, or -shSRI457 and incubated with 1µg/ml puromycin for 3 or 5 days before cell lysis and western blotting using polyclonal anti-sorcin (1:300) and monoclonal anti-tubulin (1:10,000). (F) Sorcin silencing reduces the activity of an artificial promoter containing five ATF6 binding sites in MIN6 cells. MIN6 were co transfected with p5xATF6-GL3, pRL-CMV and either pLKO.1-shScrambled, -shSRI144, or -shSRI457 for 48 or 72h before cell lysis and luciferase assay. Results are expressed as mean ± SEM, n=3 independent experiments, *p<0.005, paired two-tailed Student’s t-tests. (G-H) Quantitative RT-PCR analysis of (G) Sri and (H) spliced Xbp1: total Xbp1 were performed in MIN6 cells transduced for 24h with adenoviruses encoding sorcin-GFP or GFP only and treated with DMSO (0.1%) or thapsigargin (Thaps, 100 µM) for further 24 h. Results are expressed as mean ± SEM, n=3 independent experiments, *p<0.05, ***p<0.005, paired two-tailed Student’s t-tests.
Figure 8. Sorcin lies on a pathway linking β cell lipotoxicity to ER calcium and ER stress, representing a mechanism for dysregulation of β cell function under conditions of metabolic stress. (A) In pancreatic β-cells, sorcin is down-regulated under conditions of lipotoxic stress such as exposure to high fat diet and palmitate or pro-inflammatory cytokines as shown by others (20). The inverse relationship between sorcin and G6PC2 expression levels observed in islets suggests that an important mechanism of action of sorcin is to regulate G6PC2 expression to influence both ER stress and glucose-stimulated insulin secretion (GSIS). (B) Sorcin overexpression is sufficient to protect against β cell dysfunction during HFD. In stressed β-cells, sorcin overexpression increases ER and cytosolic [Ca\(^{2+}\)] decreasing G6PC2, through NFAT signalling pathway, which would stimulate GSIS. By maintaining a high concentration of Ca\(^{2+}\) in the ER lumen, sorcin prevents ER stress and maintains long term capacities for GSIS during HFD.
Figure 1

Figure 1
202x279mm (300 x 300 DPI)
Figure 2

210x297mm (300 x 300 DPI)
Figure 3
Figure 4
Figure 5

186x282mm (300 x 300 DPI)
Figure 7

210x297mm (300 x 300 DPI)
**Figure 8**

199x278mm (300 x 300 DPI)
Supplemental Fig. 1. Sorcin expression levels in islets of SRI-tg mice. (A-B) Total protein and mRNA were extracted from islets isolated from (A) SRI-tg1 and (B) SRI-tg10 mice (n=3-6 per genotype, 8-11 weeks old) and their respective littermate controls receiving doxycycline in the drinking water from 4 weeks old. Top panels show representative Western blots performed as in (Leclerc, et al., Am J Physiol Endocrinol Metab, 2004, 286(6):E1023-31) using rabbit polyclonal anti-sorcin (1:1000, Farrell, et al., J Biol Chem, 2003, 278(36):34660-6) and mouse monoclonal anti-alpha tubulin (1:20000, Sigma #T5168). Lower panels show qRT-PCR analysis of sorcin and luciferase expression. Values are mean ± SEM. Statistics: *p<0.05, **p<0.01, ***p<0.001, two-tailed Student’s t tests.
Supplemental Fig. 2. Sorcin phenotype is not apparent in vivo in the absence of β cell stress. (A, C) IPGTTs in SRI-tg1 male mice under standard chow diet (SD) (\(n=10-12,16\) and 24 weeks old as indicated). (B, D) IPGTTs in SRI-tg10 female mice under HFD (\(n=7-11,16\) and 24 weeks old as indicated). SRI-tg10 females, which are more resistant to diet-induced lipotoxicity than males (Oliveira et al., Ann Anat, 2015, 10.1016/j.aanat.2015.01.007), display an improved glucose tolerance only after 20 weeks of HFD. Right panels represent AUC of blood glucose concentration during IPGTTs. Values are mean ± SEM. ***\(p<0.001\); Two-way ANOVA.
Supplemental Fig. 3. Sorcin overexpression enhances GSIS in isolated islets of SRI-tg1 mice.

Ex vivo insulin secretion assays were performed on isolated islets from HFD-fed SRI-tg1 male mice \( (n=3, \text{17-week-old}) \). Mice were sacrificed by cervical dislocation and pancreatic islets were isolated by in situ collagenase digestion and cultured as previously described (Ravier, et al., Methods Mol Biol, 2010). Transgenic islets were cultured overnight in medium supplemented with 0.5µg/ml doxycycline hyclate to sustain transgene expression. Islets were divided into groups of 10 islets per condition and insulin secretion assays were performed as described in (Leclerc, et al., Am J Physiol Endocrinol Metab, 2004, 286(6):E1023-31). Secreted and total insulin content were quantified using HTRF Insulin kit (Cisbio). *p<0.05, two-tailed Student’s t tests.
Supplemental Fig. 4. Sorcin overexpression in human islets enhances glucose-induced intracellular Ca\(^{2+}\) oscillations. Cytosolic free Ca\(^{2+}\) levels ([Ca\(^{2+}\)\text{cyt}]) were measured in islets from human cadaveric donors (n=3, see Supplemental Table 2 for donors characteristics) transduced with an adenovirus encoding sorcin-GFP or GFP only. Dissociated islets were loaded with Fura-Red (Invitrogen; 4 µM) then perifused sequentially with 3 mM glucose (G3), 17 mM glucose (G17), G17 and KCl (20 mM), and G3 as indicated. Different types of responses for glucose-stimulated [Ca\(^{2+}\)\text{cyt}] increases were observed and quantified as follows: (i) no response to G17, (ii) low response to G17 (peaks > 0.3 and < 4 % over basal) and (iii) high response to G17 (peaks > 4 % over basal). (A) Representative traces of non-responsive (i), low-responsive (ii) and high-responsive (iii) islets. (B) Quantification of results. *p<0.05, Two-tailed Student’s t-tests.
Supplemental Fig. 5. Sorcin overexpression no longer enhances glucose-stimulated cytosolic Ca\(^{2+}\) levels after ER Ca\(^{2+}\) depletion. Cytosolic Ca\(^{2+}\) levels ([Ca\(^{2+}\)\(_{\text{cyt}}\)]\(_{\text{cyt}}\)) were measured in intact islets loaded with Fluo-2 MA AM (Cambridge Bioscience) from HFD-fed SRI-tg10 male mice and littermate controls (n=4, 11-week-old). The islets were perifused sequentially with 3 mM glucose (G3) in the presence of the SERCA pump inhibitor, cyclopiazonic acid (CPA, 20 \(\mu\)M, Sigma #C1530) to empty the ER, followed by 17 mM glucose (G17) glucose as indicated. Traces represent mean ± SEM. The right panel represents AUC of [Ca\(^{2+}\)]\(_{\text{cyt}}\) after glucose stimulation (time: 13-22 min). Two-tailed Student’s t-tests.
Supplemental Fig. 6. Endogenous sorcin expression in regular chow and high fat fed DBA2/J mice. Normalised RNA-Seq counts for Sri compared between regular chow and high fat diet fed mice at 30 days, six biological replicates per condition. To obtain counts, reads were mapped to mm9 and summed for all exons of a gene. The gene counts were then normalised using EdgeR and differential expression measured using a moderated t-test in limma (voom method). P-values were corrected for multiple comparisons using the Benjamini Hochberg FDR method. The estimated fold change and corrected p-value are indicated.
Supplemental Fig. 7. Effects of diazoxide and cyclosporine A on hG6PC2 promoter activity.

MIN6 β cells were co-transfected with pRL-CMV and -1075+124hG6PC2-Luci using Lipofectamine 2000 and OptiMem. After 20h (overnight) the medium was changed for 20 mM Glucose DMEM supplemented with DMSO (0.1%), diazoxide (Diaz, 100 µM) or cyclosporin A (CsA, 0.2 µM) as indicated for a further 24h before cell lysis and luciferase assays (n=3-4 independent experiments). Values are mean ± SEM. **p<0.005, ***p<0.001, Two-tailed Student’s t-tests.
Supplemental Table 1: Oligonucleotide sequences used to design pLKO.1-shSc (scrambled), pLKO.1-shSRI144, and pLKO.1-shSRI457 plasmids.

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**Supplemental Table 2**: Characteristics of donor’s providing islet samples. Details were provided by the harvesting and processing institution.

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### Supplemental Table 3: Oligonucleotide sequences of primers used for RT-qPCR.

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(1) from Lipson et al, Cell Metab. 2006 Sep;4(3):245-54.
Supplemental table 4: Ingenuity Pathway Analysis of microarray dataset. Top ranking networks (score ≥ 30); 1.5 fold-change cut-off, P-value = 0.01.

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<th>Score</th>
<th>Focus molecules</th>
<th>Up-regulated (at least 1.5 fold)</th>
<th>Down-regulated (at least 1.5 fold)</th>
<th>Top Diseases and Functions</th>
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<tr>
<td>1</td>
<td>44</td>
<td>25</td>
<td>BBS1, DHCR24*, DYNC1LI1*, KLHL13, NUDCD3*, NUP133*, SAC3D1, VANGL2</td>
<td>ALG5*, ANAPC1*, CCDC64*, CCT3*, CDC6*, CDC45, CDK1*, CENPF, HECW2, KIF1C*, LRRC59*, MAD2L1*, NEDEL1*, PDCL3, PRC1, RAD54B*, RRP1B*</td>
<td>Cell Cycle, Cellular Assembly and organization, DNA replication, Recombination and Repair</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>23</td>
<td>CLDN4, CNGA3*, DLK1*, ECE1*, ERLIN2*, HS6ST1*, IPRP3, MGAT3, PDE7A, PVRL1*, RGS3*, SRE1*, WNK4*</td>
<td>AMPH*, ATP2B4*, CCP110*, CD200*, ITPR1*, JAM2*, PDE5A*, PLCB4, RGS5, SNAP23</td>
<td>Molecular Transport, Cellular Assembly and Organization, Cellular Function and Maintenance</td>
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<td>34</td>
<td>21</td>
<td>APOF*, HIST2H2AA3/HIST2H2AA4*, HIST4H4*, INSI51*, LDLR*, LINGO1, PCOLCE2*, PRKAG3*, RAB3B*, SERPINA1*, SREBP1, ZBTB7C</td>
<td>FABP5*, Gpihbp1*, MK167, NAP1L1*, PBK, RGCC, SEMA6D*, SLC30A7*, TNFRSF10A*</td>
<td>Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism</td>
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<td>30</td>
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<td>ADRBK1*, ANXA11*, AQP7*, ASCL1*, CBLC*, DPYSL2*, FOXO3, GNA13*, GSN*, MAP15*, NOSIP*, OBFC1, SETBP1</td>
<td>CAV1, DOCK4*, KCNIP2, NOSTRIN*, NRCAM*, SDPR</td>
<td>Cancer, Organismal Injury and Abnormalities, Respiratory Disease</td>
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