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Secreted Frizzled-Related Protein 4 Reduces Insulin Secretion and is Overexpressed in Type 2 Diabetes

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SUMMARY

A plethora of candidate genes have been identified for complex polygenic disorders, but the underlying disease mechanisms remain largely unknown. We explored the pathophysiology of type-2 diabetes (T2D) by analyzing global gene expression in human pancreatic islets. A group of co-expressed genes (module), enriched for interleukin-1-related genes, was associated with T2D and reduced insulin secretion. One of the module genes, that was highly overexpressed in islets from T2D patients, is SFRP4, which encodes secreted frizzled-related protein 4. SFRP4 expression correlated with inflammatory markers, and its release from islets was stimulated by interleukin-1 β . Elevated systemic SFRP4 caused reduced glucose tolerance through decreased islet expression of Ca²⁺-channels and suppressed insulin exocytosis. SFRP4 thus provides a link between islet inflammation and impaired insulin secretion. Moreover, the protein was increased in serum from T2D patients several years before the diagnosis, suggesting that SFRP4 could be a potential biomarker for islet dysfunction in T2D.

HIGHLIGHTS

- SFRP4 is a hub gene in a T2D-associated gene co-expression module in human islets
- SFRP4 reduces glucose-induced insulin secretion through decreased β -cell exocytosis
- Expression and release of SFRP4 from islets is enhanced by interleukin-1 β
- SFRP4 is elevated in serum several years before clinical diagnosis of T2D

INTRODUCTION

Large-scale gene expression data hold great promise to provide new pathophysiological insights for a range of diseases, but there are several limitations with our current way of analyzing such information (Lander, 2011; Rosengren et al., 2010; Saxena et al., 2007; Schadt, 2009; Sladek et al., 2007; Voight et al., 2010; Zeggini et al., 2008). Network models, in which genes are represented as nodes and their connections as edges, have been proposed as a useful framework for studying complex biological data (Barabasi and Albert, 1999; Barabasi et al., 2011; Ravasz et al., 2002; Schadt, 2009; Taneera et al., 2012). To take full advantage of those models it is essential to analyze disease-relevant tissues and to combine bioinformatics with mechanistic validations. Using type-2 diabetes (T2D) as an example of a complex polygenic disorder, we have explored whether co-expression networks in human pancreatic islets could be used to identify novel disease genes.

RESULTS

Gene co-expression analysis identifies a module that is associated with T2D, elevated HbA1c and reduced insulin secretion

Impaired insulin secretion is a cardinal feature of T2D, typically combined with insulin resistance, but the underlying mechanisms are largely unknown. We obtained global microarray expression data from islets from 48 human donors, of which 10 had T2D (Table S1 available online), and investigated the gene expression topology with the weighted gene co-expression network analysis framework developed by Zhang and Horvath (Zhang and Horvath, 2005) (see the Supplemental Experimental Procedures). The connectivity, reflecting co-expression, was calculated for all pairs of gene expression traits. The resulting co-expression network exhibited the characteristic scale-free properties ($R^2 > 0.9$; Table S2) of biological networks (Ravasz et al., 2002). Next, the topological overlap, as a measure of

relative gene interconnectedness, was computed and used to identify 17 gene modules (Figure 1A).

Rather than analyzing each gene expression trait individually, the 1st principal component of the gene expression traits of each module (the “module eigengene”) was used. We identified an eigengene representing a module with 174 genes (Table S3), which had higher values in islets from diabetic donors ($p=0.010$; Table S4) and was correlated with HbA1c, reflecting long-term blood glucose, ($p=0.010$ in all donors; n.s. when analyzing only non-diabetic [ND] donors) and reduced glucose-stimulated insulin secretion ($p=0.0047$ in all donors; n.s. in ND). The corresponding module was enriched for interleukin-1-binding genes (43.3-fold enrichment; $p=0.026$) and interleukin-1 receptor activity (61.8-fold enrichment; $p=0.0079$), as well as RNA-processing genes. No other module eigengene was significantly associated with T2D (Table S4; p-values are nominal without correction for multiple testing).

We explored the possibility of using topological information to prioritize genes in the T2D-associated module. The total connectivity of a gene (the degree k) is a central parameter, as scale-free networks are characterized by a few high-degree hubs, which connect a large number of peripheral nodes (Albert et al., 2000; Carter et al., 2004; Ravasz et al., 2002).

For each gene in the T2D module we analyzed the connectivity within (k_{in}) and outside (k_{out}) the module (Zhang and Horvath, 2005). There was a significant correlation between k_{in} , but not k_{out} , and gene expression association with diabetes status (Figures 1B, 1C and 1D). We therefore selected the most connected genes in the module for further analyses. Of these, the expression of *SFRP4*, *ASAM* and *FBLN1* was most highly associated with T2D. *IL18R1*, *IL1RL1* and *SFRP4* correlated most strongly with HbA1c, and *SFRP4*, *IL18R1* and *ABCE1*

with insulin secretion. *SFRP4* was among the top three hub genes associated with all T2D traits (Figures 1E, 1F and 1G).

The network analysis was replicated in another data set using human islet microarrays from 29 donors, ten of which had T2D (Table S5). The co-expression network generated from these data also exhibited scale-free properties ($R^2 > 0.8$; Table S6), and 20 gene modules were identified (Table S7). Statistical power was lower in this smaller data set, and no module eigengene was significantly associated with T2D. However, an eigengene representing a module with 123 genes (Table S8) was nominally associated with HbA1c ($p=0.034$, all 29 donors) and showed a tendency for higher values in diabetic donors ($p=0.072$). Interestingly, the module contains *SFRP4*, as well as a high degree of shared genes with the T2D-associated module identified in the 48 donors initially analyzed ($p=1.9E-11$ using Fisher's exact test), including interleukin-1 receptors (85.9-fold enrichment). There was an association between k_{in} and gene expression association with diabetes status ($p=0.0003$) also in the replication set, and *SFRP4* was among the most highly connected genes in the module.

SFRP4 encodes secreted frizzled-related protein 4, which is a 40 kDa protein with an N-terminal cysteine-rich domain with sequence similarity with frizzled (Fz) receptors and a C-terminal netrin-like domain that has been suggested to interact with extracellular matrix proteins (Bovolenta et al., 2008; Rattner et al., 1997). *SFRP4* is an extracellular regulator of the Wntless (Wnt) pathway. Interestingly, several T2D-associated genetic variants locate to genes in the Wnt pathway (Saxena et al., 2007; Sladek et al., 2007; Voight et al., 2010; Zeggini et al., 2008), including the Wnt effector *TCF7L2* (da Silva Xavier et al., 2009; Grant et al., 2006). *SFRP4* is involved in tissue development and cancer, as well as phosphate

metabolism (Berndt et al., 2003; Bovolenta et al., 2008; Heller et al., 2002), but to date it has not been implicated in T2D.

SFRP4 reduces insulin secretion through decreased Ca^{2+} -influx and insulin exocytosis in pancreatic β -cells

We studied the effect of SFRP4 on insulin secretion by exposing isolated mouse pancreatic islets to recombinant SFRP4 for 24 h followed by 1-h stimulation with 16.7 mM glucose. The peptide dose-dependently reduced glucose-stimulated insulin secretion with maximal inhibition observed at 30 nM (Figures 2A and S1A); insulin content or β -cell viability were not affected (Figures S1B, S1C and S1D). A comparable inhibitory effect of 24-h treatment with SFRP4 on glucose-stimulated insulin secretion was also observed in human islets (Figure 2B). SFRP4 did not affect basal insulin secretion (measured at 2.8 mM glucose) in mouse (Figure 2A) or human islets (Figure 2B), and there was no association between basal insulin release and SFRP4 expression ($p=0.2$). In both human and mouse islets insulin secretion was unaffected by acute exposure to SFRP4 (Figures S1E and S1F).

Pancreatic β -cells release insulin through Ca^{2+} -dependent exocytosis, which can be monitored as increases in cell capacitance (Rorsman and Renstrom, 2003). Pretreatment with SFRP4 decreased the exocytotic responses to a train of ten depolarizations (applied to simulate glucose-induced electrical activity) in both human (Figure 2C) and mouse β -cells (Figure S1G) relative to non-treated control cells. This was paralleled by a reduction of the integrated Ca^{2+} -current (that reports the cumulative Ca^{2+} -entry) (Figures 2D and S1H). However, there was no change in the Ca^{2+} -sensitivity of the exocytotic process (defined as the capacitance increase divided by the integrated Ca^{2+} -current; Figure S1I), which suggests that the exocytosis machinery was intact. Conversely, siRNA-mediated silencing of *SFRP4* in

dispersed mouse β -cells ($63 \pm 2\%$ mRNA knock-down; $n=3$) increased Ca^{2+} -influx by 65% and exocytosis by 42% (Figure 2E) and was paralleled by a 30% reduction of accumulated SFRP4 secreted into the medium following transfection. The siRNA treatment had no effect on insulin secretion from intact mouse islets (Figure S1J), putatively due to poor transfection efficiency in the islet core, which confounds the whole-islet experiments. To specifically study the effects of SFRP4 originating from β -cells we silenced *SFRP4* in the clonal β -cell line INS832/13 ($76 \pm 6\%$ mRNA knock-down), which enhanced glucose-stimulated insulin release by 25% ($p=0.029$, one-tailed; Figures S1K and S1L).

Next, electrical activity was assessed using current-clamp recordings of mouse islet cell clusters treated with or without SFRP4. The electrical activity consisted of depolarized plateaux on which action potentials were superimposed, interrupted by periods of repolarization. There was a tendency for lower action potential amplitude and frequency in SFRP4-treated cell clusters compared with control cells (Figure S2), whilst the plateau potential did not differ between the conditions (-43 ± 1 mV without and -42 ± 1 mV with SFRP4). To specifically study the effect of SFRP4 on glucose-induced changes in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) we conducted ratiometric measurements with Fura-2. These experiments showed that the peak increase in $[\text{Ca}^{2+}]_i$ elicited by an elevation of glucose from 2.8 to 16.7 mM was reduced by $\sim 65\%$ in islets treated with SFRP4 for 24 h ($p=0.025$; Figures 2F and 2G). Furthermore, SFRP4-treated islets exhibited reduced insulin secretion both in response to 200 μM tolbutamide (which closes the ATP-sensitive K^+ -channels [K_{ATP} -channels]) and high (50 mM) K^+ (leading to membrane depolarization) (Figure 2A). Taken together, the data suggest that SFRP4 inhibits insulin secretion at a step distal to K_{ATP} -channel closure and cell depolarization through suppressed Ca^{2+} -influx.

SFRP4 affects Wnt signaling and L-type and P/Q-type Ca²⁺-channels

SFRP4 has variably been reported to activate or inhibit Wnt signaling in different tissues (Carmon and Loose, 2008; Feng et al., 2009; Gelebart et al., 2008; Lee et al., 2008; Park et al., 2008; Suzuki et al., 2004). The Wnt pathway is activated via G_{i/o}-coupled frizzled receptors (Liu et al., 2001), and inhibition of G_{i/o} proteins by pertussis toxin abolished SFRP4-mediated suppression of insulin secretion (Figure 3A). Furthermore, the ratio of unphosphorylated versus total β -catenin was elevated in SFRP4-treated mouse islets, suggestive of canonical Wnt activation (Figure 3B). Unphosphorylated β -catenin translocates to the nucleus where it affects TCF/LEF transcription factors, including *TCF7L2* (Grant et al., 2006). SFRP4 increased TCF/LEF activity (Figure 3C), confirming the stimulatory effect on Wnt signalling. Non-canonical Wnt pathways were unaffected (Figures S3A-E). SFRP4 inhibition of β -cell exocytosis was not influenced by co-treatment with canonical (Carmon and Loose, 2008) or non-canonical Wnt proteins (Ma and Wang, 2007) (Figure S3F).

TCF/LEF activation has been shown to repress Ca²⁺-channel expression (Wisniewska et al.), and we investigated whether SFRP4-induced suppression of insulin release involved altered expression of Ca²⁺-channels. Notably, both L-type (Ca_v1.2 and Ca_v1.3) and P/Q-type (Ca_v2.1) Ca²⁺-channels were downregulated by SFRP4 in human β -cells (Figure S4A). T-type Ca²⁺-channels (Ca_v3.2) were unaffected. We next analyzed the effect of the L-type Ca²⁺-channel blocker isradipine and the P/Q-type blocker ω -agatoxin IVA (Braun et al., 2008) on the current-voltage (I-V) relationship in human β -cells incubated with or without SFRP4 for 24 h. SFRP4-treated cells displayed a pronounced reduction of the peak current (~55% at 0 mV; $p=3.5E-5$; Figure 3D). Interestingly, while isradipine suppressed the current in control cells ($p=0.028$ at 0 mV), it was without effect in SFRP4-treated β -cells (Figure 3E). Likewise,

ω -agatoxin IVA decreased the peak current in control cells ($p=0.001$ at 0 mV), but was largely ineffective in SFRP4-treated β -cells, except a tendency ($p=0.3$) for a suppression at 0 mV (~25% compared with the ~55% reduction in control cells). These findings suggest that both L-type and P/Q-type Ca^{2+} -channels are suppressed by SFRP4. Indeed, the expression of *SFRP4* in human islets was negatively correlated with *CACNA1A* ($\text{Ca}_v2.1$) ($p=0.008$; $r=-0.376$) and *CACNA1D* ($\text{Ca}_v1.3$) mRNA ($p=0.003$; $r=-0.426$).

SFRP4 expression and release is stimulated by interleukin-1 β

The regulation of SFRP4 production and release has not been characterized. Immunohistochemistry demonstrated SFRP4 in both α - and β -cells without preferential accumulation in the secretory granules (Figures 3F and S4B). Stimulation for 1 h with 16.7 mM glucose or 50 mM K^+ to elicit depolarization-induced exocytosis did not affect SFRP4 release, indicating that SFRP4 is released in a constitutive fashion rather than co-secreted with insulin (Figures S4C and S4D).

There was no significant association between known T2D gene variants and *SFRP4* expression (Table S9). Since the T2D module containing *SFRP4* was enriched for interleukin-1-related genes, we hypothesized that SFRP4 might be an inflammatory mediator. Interestingly, human islets incubated with interleukin-1 β (IL-1 β) displayed a 1.8-fold elevation of *SFRP4* mRNA (Figure 3G) and a three-fold higher secretion of the protein into the medium (Figure 3H). Moreover, 24 h treatment of human islets with 100 μM YVAD-CMK, an inhibitor of interleukin-1 β convertase that cleaves and activates the IL-1 β precursor (Samad et al., 2001), or recombinant human interleukin-1 receptor antagonist (500 ng/ml) reduced SFRP4 secretion (Figures S4E and S4F). SFRP4 expression correlated with islet content of IL-6 ($p=5\text{E-}5$; $r=0.58$) and IL-8 ($p=0.001$; $r=0.52$). By contrast, IL-1 β , IL-6 and

IL-8 expression was not affected by SFRP4 exposure (Figures 3G, S4G and S4H), which suggests that SFRP4 acts downstream of IL-1 β to reduce insulin secretion. Indeed, analysis of transcription factor binding sites near *SFRP4* (see Supplemental Experimental Procedures) demonstrated several putative binding sites for NF- κ B, which is elevated by IL-1 β (Figure 3G). Moreover, the inhibitory effect of IL-1 β on insulin secretion was attenuated ($p=0.001$) in INS832/13 cells transfected with siRNA against *SFRP4* compared with cells treated with an inactive control siRNA (Figure S4I). Due to incomplete *SFRP4* knock-down ($76\pm6\%$) it is however difficult to estimate the exact extent by which SFRP4 contributes to the IL-1 β effect.

SFRP4 injections *in vivo* reduce insulin secretion in mice and SFRP4 is elevated in serum from T2D patients

Next, mice were injected three times during 24 h with SFRP4, which resulted in glucose intolerance and reduced insulin secretion (Figure 4A). Interestingly, isolated islets from SFRP4-treated mice displayed decreased glucose-induced insulin secretion as well as suppressed β -cell Ca^{2+} -influx and exocytosis (Figures 4B and 4C). This demonstrates that elevated SFRP4 *in vivo* induces sustained inhibitory effects on β -cell function.

Finally, we investigated SFRP4 concentration in serum from 88 individuals (Groop et al., 1996) who were analyzed at three different visits (median interval 3 years) (Table S10). Serum levels of SFRP4 were significantly elevated in T2D patients ($p=0.018$, age-, sex- and BMI-corrected; $n=23$) compared with non-diabetic subjects ($n=51$) (Figure 4D). Interestingly, non-diabetic individuals who later developed T2D (“converters”; $n=14$) had significantly higher SFRP4 already at the initial visits and prior to presentation of the disease ($p=0.046$) (Figure 4E). Among non-diabetic individuals with SFRP4 levels below the median (23.7

ng/ml) 9% (3 out of 35 subjects) developed T2D at a later stage, whilst 37% (11 out of 30) of those with SFRP4 above the median developed the disease (Table S11). The odds ratio (OR) was 5.35 (95% CI: 1.29-22.2; $p=0.021$, age-, sex- and BMI-corrected).

These analyses were replicated in an extended cohort with 69 converters and 69 matched controls, in which serum SFRP4 was analyzed and an oral glucose tolerance test (OGTT) conducted at two consecutive visits (median interval 4 years [range 1-15 years]) (Table S12). The converters had been classified as non-diabetic at the first visit but were diagnosed with T2D at the second visit. In non-diabetic individuals, SFRP4 was associated with higher fasting glucose ($p=0.004$; $\beta=0.142$), reduced insulin sensitivity index ($p=0.002$; $\beta=-0.176$) and lower disposition index (insulin secretion adjusted for insulin sensitivity; $p=0.029$; $\beta=-0.186$; Table S13). Serum concentration was not affected by BMI, sex, patient or sample age (Table S13). Supporting the results from the initial analysis, SFRP4 was significantly elevated in T2D individuals by the time of diagnosis (the second visit) compared with controls (39% increase; one-tailed $p=0.044$ and $\beta=0.164$ with age, sex and BMI correction; Figure 4F). Interestingly, and further corroborating the initial findings, converters had increased SFRP4 already at their first visit, prior to clinical diagnosis (37% increase compared with controls; one-tailed $p=0.034$ and $\beta=0.163$ with correction for age, sex and BMI). The p -values were 0.12 and 0.07 (one-tailed), respectively, if adjusting for fasting glucose and glucose at 2 hr during the OGTT, which suggests that the effect is largely driven by the association between SFRP4 and glucose levels. We next studied the usefulness of categorizing individuals into quartiles based on serum SFRP4 at the first visit. As shown in Figures 4G and 4H, individuals with SFRP4 in the upper quartile were at particular risk of developing T2D ($p=0.005$; OR 3.32 [95% CI: 1.34-8.24]) compared with subjects in quartiles 1-3. The risk elevation was significant even after correction for fasting glucose ($p=0.018$;

OR=2.89 [95% CI: 1.07-7.85]) or glucose at 2 hr during the OGTT (p=0.022; OR=3.05 [95% CI: 1.04-9.0]). Further study is needed to assess if SFRP4 can be used as a biomarker for T2D risk independent of glucose.

DISCUSSION

The study identifies SFRP4 as a novel molecular link between islet inflammation and defective insulin secretion. It also demonstrates the utility of modular analyses of biological data to find disease genes that are not obvious candidates from genetic association studies, although it is difficult to establish causality from expression studies with restricted number of subjects. SFRP4 affects Wnt signaling, which influences a wide scope of genes, here reflected by the suppression of two different voltage-gated Ca^{2+} -channels that in turn lead to reduced insulin exocytosis. Indeed, the main effect of SFRP4 in β -cells was the reduction of insulin secretion stimulated by glucose and other secretagogues, whilst insulin content and β -cell viability were unaltered. It is of interest that the Wnt pathway has recently been implicated in the pathogenesis of T2D from the identification of several T2D-associated DNA variants that locate near genes in the Wnt pathway (Saxena et al., 2007; Sladek et al., 2007; Voight et al., 2010; Zeggini et al., 2008), most notably the Wnt effector *TCF7L2* (da Silva Xavier et al., 2009; Grant et al., 2006).

Our data demonstrate that SFRP4 is present in both α - and β -cells and is released from islets, probably in a constitutive fashion. The gene co-expression module containing *SFRP4* was enriched for interleukin-1-related genes, and we found that the expression and release of SFRP4 from islets was stimulated by IL-1 β . Interestingly, analysis of β -cell-enriched areas of pancreatic sections by laser-capture microdissection has shown that several inflammatory genes which are induced by IL-1 β are overexpressed in T2D (Igoillo-Esteve et al., 2010).

Moreover, IL-1 β antagonism improves glycemia and β -cell function (Cavelti-Weder et al., 2012; Dinarello et al., 2010; Larsen et al., 2007). The present data explain previous observations that IL-1 β inhibits glucose-stimulated Ca²⁺-uptake and insulin release by a pertussis toxin-sensitive mechanism (Helqvist et al., 1989; Rabuazzo et al., 1995; Thaik et al., 1995). Since SFRP4 is associated with several inflammatory mediators in human islets, it can however not be excluded that other cytokines in addition to IL-1 β also affect SFRP4 release.

Secreted frizzled-related proteins (SFRPs) have a C-terminal netrin-like domain, which binds heparin and heparan sulfate proteoglycans in the extracellular matrix and facilitates the accumulation of SFRPs at high local concentrations at the site of secretion (Bafico et al., 1999; Salic et al., 1997; Uren et al., 2000). This makes it likely that the intraislet concentration of SFRP4 is considerably higher than that in serum, and our measurements of the amount of SFRP4 released from islets (~1 pg/islet during 24 h; Figure S4F) clearly suggest that the concentration used in the experiments (30 nM) is in the physiological range.

SFRP4 in serum was associated with elevated fasting glucose and reduced disposition index. However, it was also associated with impaired insulin sensitivity, indicating that the protein could have a plethora of metabolic effects and might be released from several tissues involved in glucose homeostasis. It is interesting to note from the siRNA experiments targeting SFRP4 that also relatively modest changes in the levels of released SFRP4 (~30% reduction using siRNA in mouse islets) can cause pronounced alterations of islet function. The elevation of SFRP4 in serum several years before the diagnosis highlights the protein as a potential biomarker for the low-grade islet inflammation that is commonly seen in T2D, and it might prove useful for early disease detection in combination with glucose and other biomarkers.

Finally, our data raise the exciting possibility that SFRP4 could be a novel therapeutic target for specific treatment of islet dysfunction.

EXPERIMENTAL PROCEDURES

Human islets

Experimental procedures were approved by the local ethical committees. Donated human pancreatic islets were obtained (with research consent) from the Nordic Network for Clinical Islet Transplantations (Prof. O. Korsgren). Donors with known T2D or HbA_{1c} > 6.0% and no GAD antibodies were defined as having T2D. There was no difference in islet purity between non-diabetic and T2D donors. See Tables S1 and S5 for data on donors. All human islet microarray data are MIAME compliant, and the raw data have been deposited in a MIAME database (GEO, accession number: GSE38642).

Insulin secretion and exocytosis measurements

Insulin secretion from isolated islets and β -cell exocytosis were measured as previously described (Rosengren et al., 2010).

Pancreatic islet cell viability

Islet cell viability was measured using an Aqueous One Solution Cell Proliferation Assay Reagent (Promega, Sweden) according to the manufacturer's instructions.

RNA interference

Islets or cell lines were transfected with siRNAs using Lipofectamine RNAiMax (Invitrogen, Sweden) and totally 45 nM oligonucleotides.

TCF/LEF activity

TCF/LEF activity in INS832/13-cells was measured with the Wnt Cignal™ Reporter Assay Kit (SABiosciences, USA) according to the manufacturer's instructions.

[Ca²⁺]_i measurements

[Ca²⁺]_i was estimated by Fura-2 and dual-wavelength fluorimetry.

SFRP4 content

SFRP4 in serum and islet incubation medium was measured with a human SFRP4 ELISA kit (Nordic Diagnostica, Sweden) according to the manufacturer's instructions.

Quantitative PCR

RNA was extracted with chloroform precipitation using the mRNeasy kit (Qiagen). Gene expression was measured by qPCR using TaqMan (Applied Biosystems).

In vivo experiments

Female NMRI mice (30 g) were injected intraperitoneally with SFRP4 (200 µg/kg in PBS) or PBS at 24, 16 and 8 h before intravenous glucose tolerance tests (IVGTT). IVGTT was performed after 4 h fasting.

Individuals from the Botnia study

Serum samples were obtained from individuals (88 in the initial analysis and 138 in the replication) participating in the Botnia Study (Groop et al., 1996). Individuals between 18 and 70 years were invited to prospective visits.

Statistical analyses

Student's *t*-test was used for comparisons of data from the cellular and animal experiments. For experiments using islets or β-cells from human donors the average for each donor under the different conditions was used in the analyses and compared with paired Student's *t*-test. One-sided tests were used for the replication analyses, and two-sided tests were used otherwise.

The SFRP4 concentration in serum showed non-Gaussian distribution. Gaussian distribution was obtained using logarithm transformation. All statistical analyses of serum SFRP4 were therefore performed using ln-transformed data. All statistical analyses were performed using IBM SPSS Statistics (ver 20.0).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, 4 figures, and 13 tables and can be found with this article online.

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REFERENCES

- Albert, R., Jeong, H., and Barabasi, A.L. (2000). Error and attack tolerance of complex networks. *Nature* 406, 378-382.
- Bafico, A., Gazit, A., Pramila, T., Finch, P.W., Yaniv, A., and Aaronson, S.A. (1999). Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. *J Biol Chem* 274, 16180-16187.
- Barabasi, A.L., and Albert, R. (1999). Emergence of scaling in random networks. *Science* 286, 509-512.
- Barabasi, A.L., Gulbahce, N., and Loscalzo, J. (2011). Network medicine: a network-based approach to human disease. *Nat Rev Genet* 12, 56-68.

Berndt, T., Craig, T.A., Bowe, A.E., Vassiliadis, J., Reczek, D., Finnegan, R., Jan De Beur, S.M., Schiavi, S.C., and Kumar, R. (2003). Secreted frizzled-related protein 4 is a potent tumor-derived phosphaturic agent. *J Clin Invest* 112, 785-794.

Bovolenta, P., Esteve, P., Ruiz, J.M., Cisneros, E., and Lopez-Rios, J. (2008). Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. *J Cell Sci* 121, 737-746.

Braun, M., Ramracheya, R., Bengtsson, M., Zhang, Q., Karanauskaite, J., Partridge, C., Johnson, P.R., and Rorsman, P. (2008). Voltage-gated ion channels in human pancreatic beta-cells: electrophysiological characterization and role in insulin secretion. *Diabetes* 57, 1618-1628.

Carmon, K.S., and Loose, D.S. (2008). Secreted frizzled-related protein 4 regulates two Wnt7a signaling pathways and inhibits proliferation in endometrial cancer cells. *Mol Cancer Res* 6, 1017-1028.

Carter, S.L., Brechbuhler, C.M., Griffin, M., and Bond, A.T. (2004). Gene co-expression network topology provides a framework for molecular characterization of cellular state. *Bioinformatics* 20, 2242-2250.

Cavelti-Weder, C., Babians-Brunner, A., Keller, C., Stahel, M.A., Kurz-Levin, M., Zayed, H., Solinger, A.M., Mandrup-Poulsen, T., Dinarello, C.A., and Donath, M.Y. (2012). Effects of gevokizumab on glycemia and inflammatory markers in type 2 diabetes. *Diabetes Care* 35, 1654-1662.

da Silva Xavier, G., Loder, M.K., McDonald, A., Tarasov, A.I., Carzaniga, R., Kronenberger, K., Barg, S., and Rutter, G.A. (2009). TCF7L2 regulates late events in insulin secretion from pancreatic islet beta-cells. *Diabetes* 58, 894-905.

Dinarello, C.A., Donath, M.Y., and Mandrup-Poulsen, T. (2010). Role of IL-1beta in type 2 diabetes. *Curr Opin Endocrinol Diabetes Obes* 17, 314-321.

Feng, C., Araki, M., Kunitomo, R., Tamon, A., Makiguchi, H., Nijima, S., Tsujimoto, G., and Okuno, Y. (2009). GEM-TREND: a web tool for gene expression data mining toward relevant network discovery. *BMC Genomics* 10, 411.

Gelebart, P., Anand, M., Armanious, H., Peters, A.C., Dien Bard, J., Amin, H.M., and Lai, R. (2008). Constitutive activation of the Wnt canonical pathway in mantle cell lymphoma. *Blood* 112, 5171-5179.

Grant, S.F., Thorleifsson, G., Reynisdottir, I., Benediktsson, R., Manolescu, A., Sainz, J., Helgason, A., Stefansson, H., Emilsson, V., Helgadóttir, A., *et al.* (2006). Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. *Nat Genet* 38, 320-323.

Groop, L., Forsblom, C., Lehtovirta, M., Tuomi, T., Karanko, S., Nissen, M., Ehrnstrom, B.O., Forsen, B., Isomaa, B., Snickars, B., *et al.* (1996). Metabolic consequences of a family history of NIDDM (the Botnia study): evidence for sex-specific parental effects. *Diabetes* 45, 1585-1593.

Heller, R.S., Dichmann, D.S., Jensen, J., Miller, C., Wong, G., Madsen, O.D., and Serup, P. (2002). Expression patterns of Wnts, Frizzleds, sFRPs, and misexpression in transgenic mice suggesting a role for Wnts in pancreas and foregut pattern formation. *Dev Dyn* 225, 260-270.

Helqvist, S., Bouchelouche, P., Andersen, H.U., and Nerup, J. (1989). Modulation of calcium flux influences interleukin 1 beta effects on insulin release from isolated islets of Langerhans. *Acta Endocrinol (Copenh)* 121, 447-455.

Igoillo-Esteve, M., Marselli, L., Cunha, D.A., Ladriere, L., Ortis, F., Grieco, F.A., Dotta, F., Weir, G.C., Marchetti, P., Eizirik, D.L., *et al.* (2010). Palmitate induces a pro-inflammatory response in human pancreatic islets that mimics CCL2 expression by beta cells in type 2 diabetes. *Diabetologia* 53, 1395-1405.

Lander, E.S. Initial impact of the sequencing of the human genome. (2011). *Nature* 470, 187-197.

Larsen, C.M., Faulenbach, M., Vaag, A., Volund, A., Ehses, J.A., Seifert, B., Mandrup-Poulsen, T., and Donath, M.Y. (2007). Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *N Engl J Med* 356, 1517-1526.

Lee, S.H., Demeterco, C., Geron, I., Abrahamsson, A., Levine, F., and Itkin-Ansari, P. (2008). Islet specific Wnt activation in human type II diabetes. *Exp Diabetes Res* 2008, 728763.

Liu, T., DeCostanzo, A.J., Liu, X., Wang, H., Hallagan, S., Moon, R.T., and Malbon, C.C. (2001). G protein signaling from activated rat frizzled-1 to the beta-catenin-Lef-Tcf pathway. *Science* 292, 1718-1722.

Ma, L., and Wang, H.Y. (2007). Mitogen-activated protein kinase p38 regulates the Wnt/cyclic GMP/Ca²⁺ non-canonical pathway. *J Biol Chem* 282, 28980-28990.

Park, J.R., Jung, J.W., Lee, Y.S., and Kang, K.S. (2008). The roles of Wnt antagonists Dkk1 and sFRP4 during adipogenesis of human adipose tissue-derived mesenchymal stem cells. *Cell Prolif* 41, 859-874.

Rabuazzo, A.M., Buscema, M., Caltabiano, V., Anello, M., Degano, C., Patane, G., Vigneri, R., and Purrello, F. (1995). Interleukin-1 beta inhibition of insulin release in rat pancreatic islets: possible involvement of G-proteins in the signal transduction pathway. *Diabetologia* 38, 779-784.

Rattner, A., Hsieh, J.C., Smallwood, P.M., Gilbert, D.J., Copeland, N.G., Jenkins, N.A., and Nathans, J. (1997). A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proc Natl Acad Sci U S A* 94, 2859-2863.

Ravasz, E., Somera, A.L., Mongru, D.A., Oltvai, Z.N., and Barabasi, A.L. (2002). Hierarchical organization of modularity in metabolic networks. *Science* 297, 1551-1555.

Rorsman, P., and Renstrom, E. (2003). Insulin granule dynamics in pancreatic beta cells. *Diabetologia* 46, 1029-1045.

Rosengren, A.H., Jokubka, R., Tojjar, D., Granhall, C., Hansson, O., Li, D.Q., Nagaraj, V., Reinbothe, T.M., Tuncel, J., Eliasson, L., *et al.* (2010). Overexpression of alpha2A-adrenergic receptors contributes to type 2 diabetes. *Science* 327, 217-220.

Salic, A.N., Kroll, K.L., Evans, L.M., and Kirschner, M.W. (1997). Sizzled: a secreted Xwnt8 antagonist expressed in the ventral marginal zone of *Xenopus* embryos. *Development* 124, 4739-4748.

Samad, T.A., Moore, K.A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J.V., and Woolf, C.J. (2001). Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 410, 471-475.

Saxena, R., Voight, B.F., Lyssenko, V., Burtt, N.P., de Bakker, P.I., Chen, H., Roix, J.J., Kathiresan, S., Hirschhorn, J.N., Daly, M.J., *et al.* (2007). Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* 316, 1331-1336.

Schadt, E.E. (2009). Molecular networks as sensors and drivers of common human diseases. *Nature* 461, 218-223.

Sladek, R., Rocheleau, G., Rung, J., Dina, C., Shen, L., Serre, D., Boutin, P., Vincent, D., Belisle, A., Hadjadj, S., *et al.* (2007). A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* 445, 881-885.

Suzuki, H., Watkins, D.N., Jair, K.W., Schuebel, K.E., Markowitz, S.D., Chen, W.D., Pretlow, T.P., Yang, B., Akiyama, Y., Van Engeland, M., *et al.* (2004). Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 36, 417-422.

Taneera, J., Lang, S., Sharma, A., Fadista, J., Zhou, Y., Ahlqvist, E., Jonsson, A., Lyssenko, V., Vikman, P., Hansson, O., *et al.* (2012). A systems genetics approach identifies genes and pathways for type 2 diabetes in human islets. *Cell Metab* 16, 122-134.

Thaik, C.M., Calderone, A., Takahashi, N., and Colucci, W.S. (1995). Interleukin-1 beta modulates the growth and phenotype of neonatal rat cardiac myocytes. *J Clin Invest* 96, 1093-1099.

Uren, A., Reichsman, F., Anest, V., Taylor, W.G., Muraio, K., Bottaro, D.P., Cumberledge, S., and Rubin, J.S. (2000). Secreted frizzled-related protein-1 binds directly to Wingless and is a biphasic modulator of Wnt signaling. *J Biol Chem* 275, 4374-4382.

Voight, B.F., Scott, L.J., Steinthorsdottir, V., Morris, A.P., Dina, C., Welch, R.P., Zeggini, E., Huth, C., Aulchenko, Y.S., Thorleifsson, G., *et al.* (2010). Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat Genet* 42, 579-589.

Wisniewska, M.B., Misztal, K., Michowski, W., Szczot, M., Purta, E., Lesniak, W., Klejman, M.E., Dabrowski, M., Filipkowski, R.K., Nagalski, A., *et al.* (2010). LEF1/beta-catenin complex regulates transcription of the Cav3.1 calcium channel gene (*Cacna1g*) in thalamic neurons of the adult brain. *J Neurosci* 30, 4957-4969.

Zeggini, E., Scott, L.J., Saxena, R., Voight, B.F., Marchini, J.L., Hu, T., de Bakker, P.I., Abecasis, G.R., Almgren, P., Andersen, G., *et al.* (2008). Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nat Genet* 40, 638-645.

Zhang, B., and Horvath, S. (2005). A general framework for weighted gene co-expression network analysis. *Stat Appl Genet Mol Biol* 4, Article17.

FIGURE LEGENDS

Figure 1. Topological characteristics and association between gene expression and T2D traits.

(A) Symmetrically arranged heatmap of the topological overlap matrix for which the rows and columns are sorted by the hierarchical clustering tree used to define modules. Intensity from light yellow (low) to red (high) denotes the topological overlap between gene pairs. The red circle indicates the T2D-associated module.

(B-D) For each of the 174 genes in the T2D module the absolute value of the correlation of the gene expression trait and diabetes status (B), HbA1c (C), or glucose-stimulated insulin secretion (D) is displayed against the logarithm of k_{in} for the gene. Red dots indicate SFRP4. P- and r-values for the correlation between k_{in} and gene expression association with the diabetes traits were calculated by Spearman's rank correlation.

(E) SFRP4 expression (log2-transformed) in human islets from non-diabetic (n=38) and diabetic (n=10) donors. P-value was calculated using logistic regression.

(F) SFRP4 expression in human islets plotted against HbA1c in the donors (n=38).

(G) SFRP4 expression plotted versus insulin secretion at 16.7 mM glucose in human islets (n=35).

P-values in (F) and (G) were calculated using linear regression and are given for all donors as well as only non-diabetic (ND) donors, for which n=28 in (F) and n=26 in (G).

Figure 2. Effects of SFRP4 on insulin secretion and β -cell exocytosis.

(A) Insulin secretion in response to 1-h incubations with 2.8 mM glucose, 16.7 mM glucose, 2.8 mM glucose plus 50 mM K^+ , or 8.3 mM glucose plus 200 μ M tolbutamide in mouse islets that were cultured with or without 30 nM SFRP4 for 24 h prior to experiments. SFRP4 was not present during the 1-h incubations. Data from 6-12 experiments per group. P-values from unpaired comparisons.

(B) Insulin secretion in response to 1-h incubations with 2.8 or 16.7 mM glucose in human islets cultured in the absence or presence of 30 nM SFRP4 for 24 h. Data from 5-8 experiments per group from each of 3 donors at 2.8 mM glucose, and data from 4-12 experiments per group from each of 17 donors at 16.7 mM glucose. P-values from paired comparisons for each donor.

(C) Increase in cell capacitance (ΔC), reflecting exocytosis, evoked by a train of ten 500 ms depolarizations from -70 to 0 mV applied to a human β -cell to simulate glucose-induced electrical activity. Histogram shows total capacitance increase ($\Sigma\Delta C$) in human β -cells pre-incubated without or with 30 nM SFRP4 for 24 h. SFRP4 was not present in the extracellular solution during the recordings. Data from 3-11 experiments per donor from 16 donors with paired comparisons.

(D) The Ca^{2+} -current (I) in response to the first depolarization of the train. The bars denote the integrated Ca^{2+} -current (charge) in human β -cells pre-incubated without or with SFRP4. Data from same cells as in (C).

(E) Left histogram shows average total capacitance increase in response to a train stimulus in mouse β -cells treated with control siRNA or siRNA against SFRP4. Right bars show the mean integrated Ca^{2+} -current (charge) in response to the first depolarization. Data from 17 and 39 cells, respectively. P-values from unpaired one-sided tests.

(F) Representative recordings of Fura-2 fluorescence at 340 vs. 380 nm ($\Delta F_{340}/F_{380}$ from the average baseline) in mouse islets that had been incubated for 24 h in the absence or presence of 30 nM SFRP4. The extracellular glucose concentration was changed from 2.8 to 16.7 mM during the experiment as indicated. Sampling rate is 0.2 Hz.

(G) The histogram shows the mean $\Delta F_{340}/F_{380}$ peak at high glucose in control (n=5) and SFRP4-treated islets (n=5). Data are mean $\pm$ s.e.m. *p<0.05; **p<0.01; ***p<0.001.

Figure 3. Regulation of SFRP4 and effects in β -cells.

(A) Insulin secretion in response to 16.7 mM glucose in mouse islets pre-treated with or without 100 ng/ml pertussis toxin (PTX) or 30 nM SFRP4 as indicated (n=6 per group).

(B) Immunoblots of total protein from mouse islets incubated for 24 h in the absence or presence of SFRP4. Polyclonal antisera against actin, total β -catenin and unphosphorylated β -catenin were used as indicated. The histogram shows average levels of unphosphorylated versus total β -catenin (n=6 blots).

(C) Activity of an inducible TCF/LEF-responsive reporter in INS832/13-cells incubated with or without SFRP4. Data from 3 experiments with 5 wells per experiment and group.

(D) Current-voltage relationship for the total Ca^{2+} -current in the absence or presence of 2 μM isradipine or 200 nM ω -agatoxin IVA in control or SFRP4-treated human β -cells (n=3-15 cells per group).

(E) L- and P/Q-type Ca^{2+} -currents obtained by subtracting currents recorded in the presence of isradipine or ω -agatoxin IVA from currents observed in the absence of the respective antagonist. The cells were pre-incubated without or with 30 nM SFRP4 for 24 h.

(F) Immunostaining of human β -cell with anti-insulin (red) and anti-SFRP4 (blue) antibodies. Lower right shows overlay. Scale bars 2 μm .

(G) Human islet mRNA expression of indicated genes. Islets were incubated with or without 25 ng/ml IL-1 β or 30 nM SFRP4 as shown (n=8 donors).

(H) SFRP4 content in 2 ml medium with 100 islets incubated with or without 25 ng/ml IL-1 β for 24 h (n=4 experiments).

Data are mean $\pm$ s.e.m. *p<0.05; **p<0.01; ***p<0.001.

Figure 4. *In vivo* effects of SFRP4 and SFRP4 in human serum.

(A) Glucose and insulin levels during an intravenous glucose tolerance test (IVGTT). PBS or SFRP4 (200 $\mu\text{g/kg}$) were injected 24, 16 and 8 h before the IVGTT. Data from 12 control and 13 SFRP4-treated mice.

(B) Insulin secretion *in vitro* in response to a 1-h incubation at 16.7 mM glucose from islets isolated from PBS- or SFRP4-injected mice. Data from 8 (PBS) and 10 (SFRP4) experiments, respectively.

(C) Increase in cell capacitance (ΔC) evoked by a depolarization train in β -cells from mice injected with PBS or SFRP4. The histograms show the average total capacitance increase

($\Sigma\Delta C$) and integrated Ca^{2+} -current (charge). Data from 13 and 16 cells from control and SFRP4-treated mice, respectively. Islets were not exposed to SFRP4 after isolation from the animals for experiments in (B) and (C).

(D) Serum concentration of SFRP4 in individuals who remained non-diabetic at all visits (ND; n=51 individuals) and subjects with T2D at all visits (T2D; n=23 individuals).

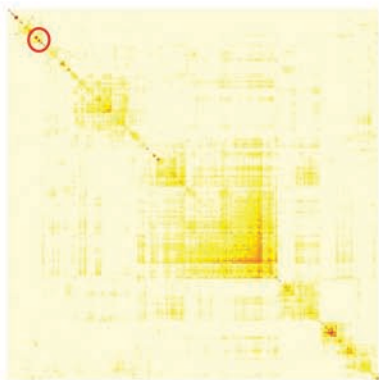
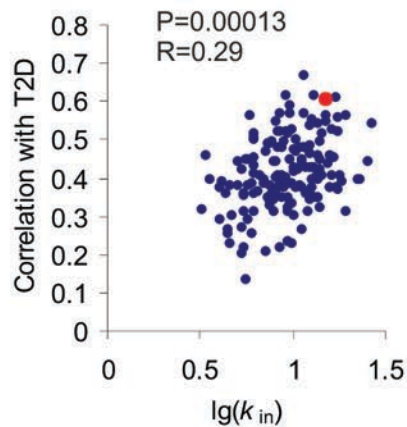
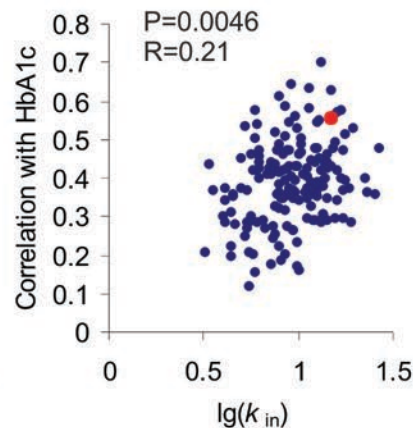
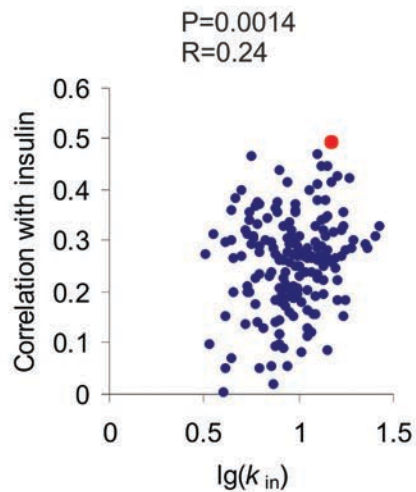
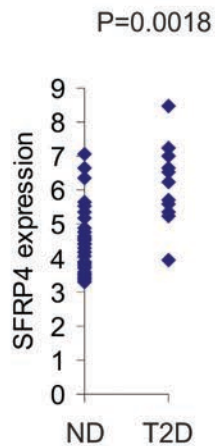
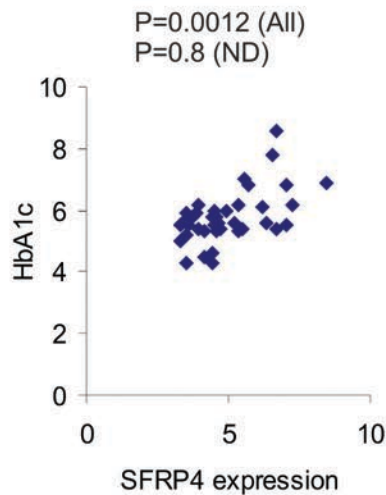
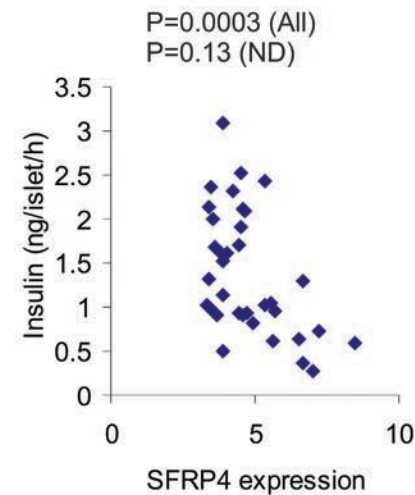
(E) Serum SFRP4 measured at visit 1 and 2 in non-diabetic individuals who later developed T2D (converters; n=14 individuals) compared with subjects who remained non-diabetic (ND; p-value from one-sided comparisons corrected for age, sex and BMI).

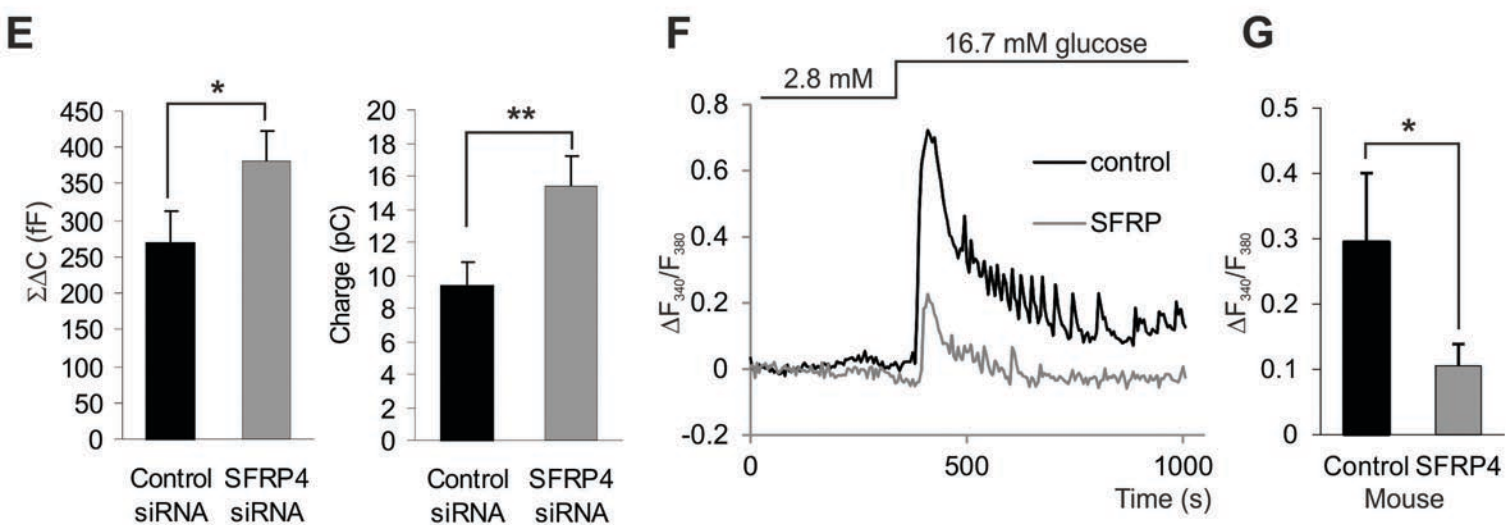
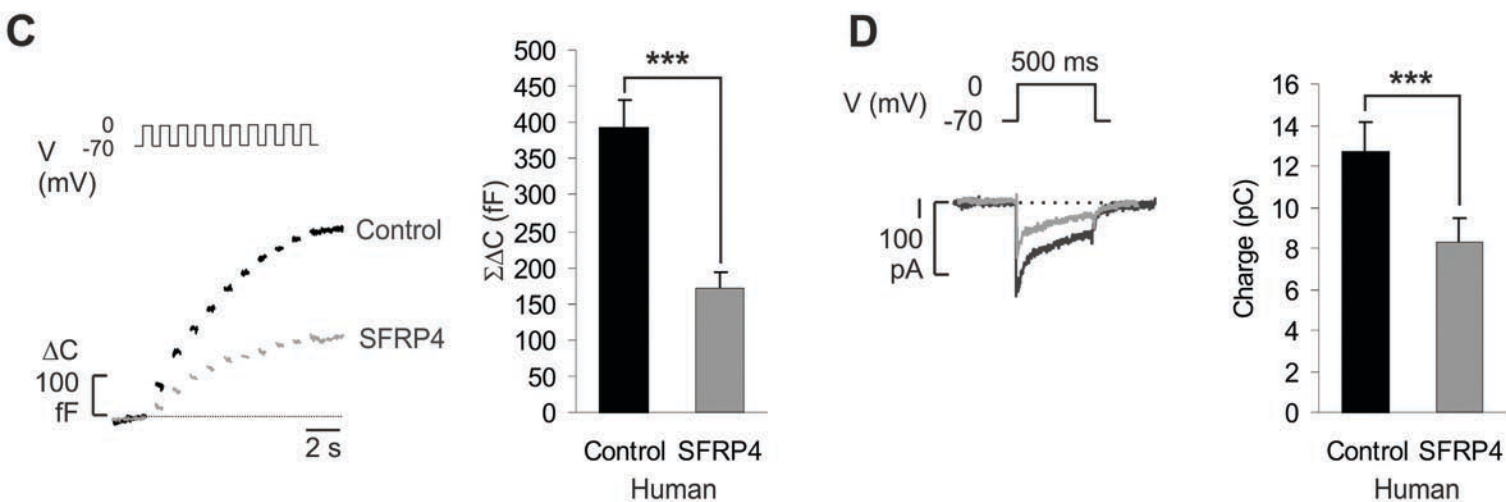
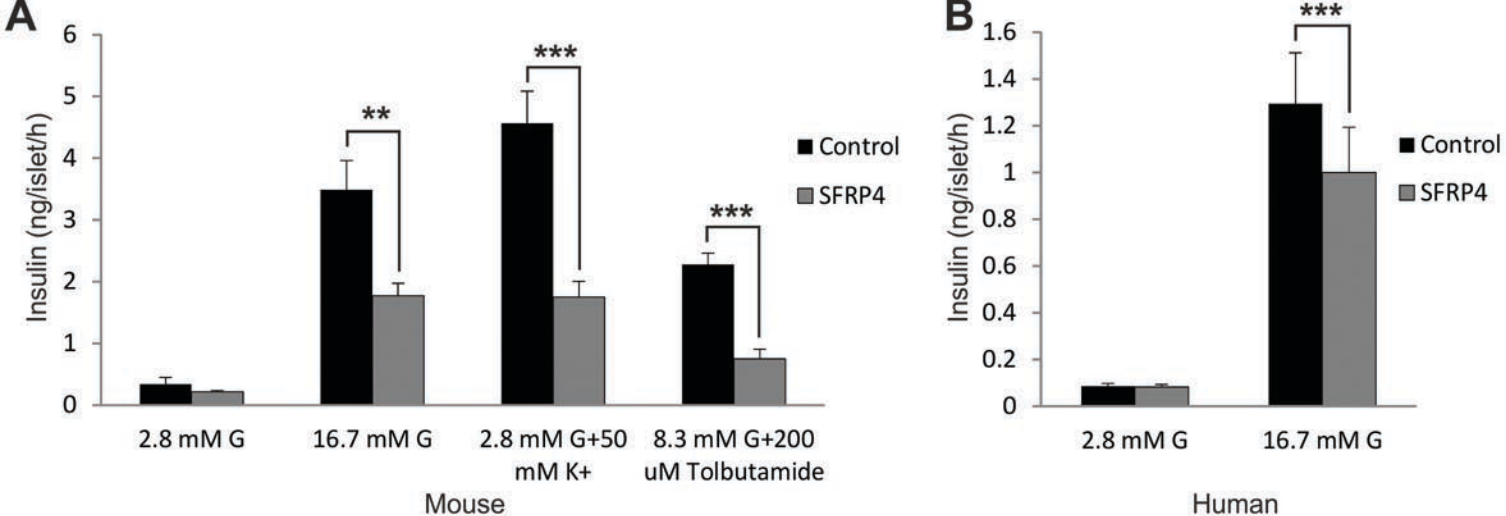
(F) Serum SFRP4 measured in individuals who were non-diabetic at both visit 1 (n=61) and 2 (n=64) and converters who were non-diabetic at visit 1 (n=62) and diagnosed with T2D at visit 2 (n=60).

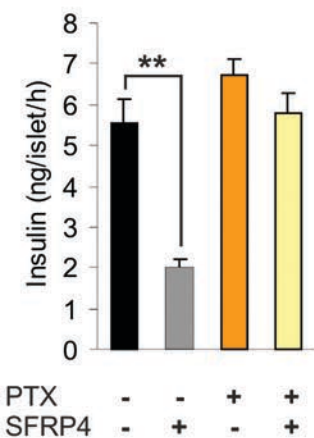
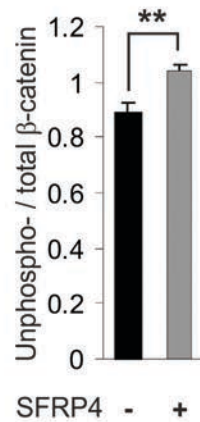
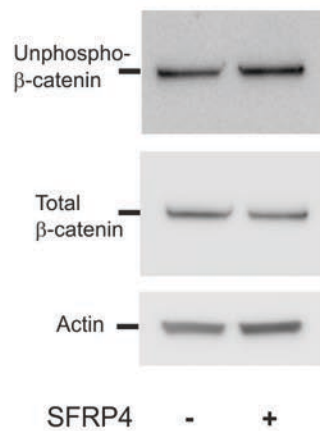
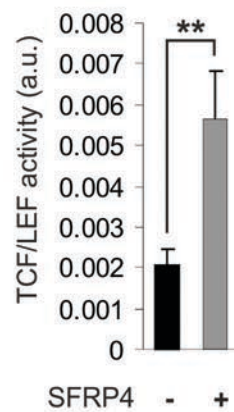
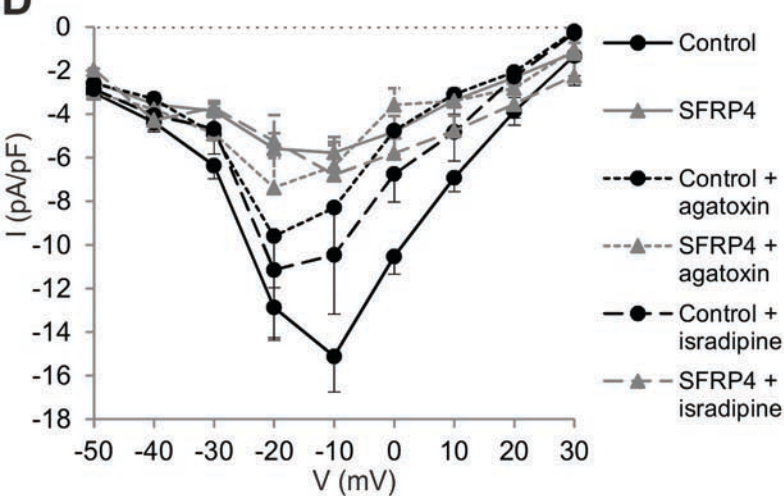
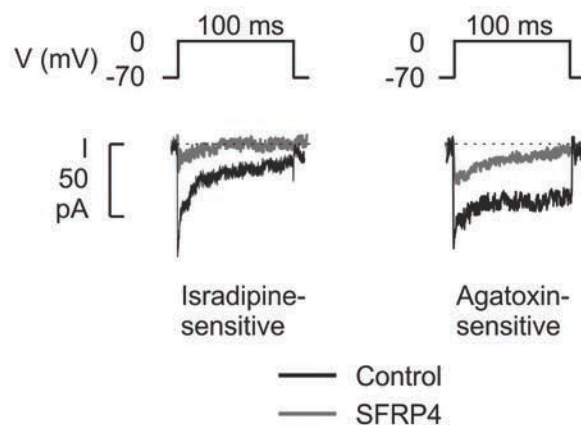
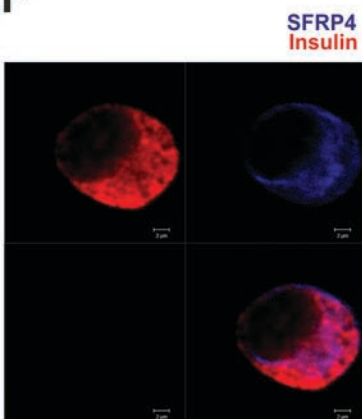
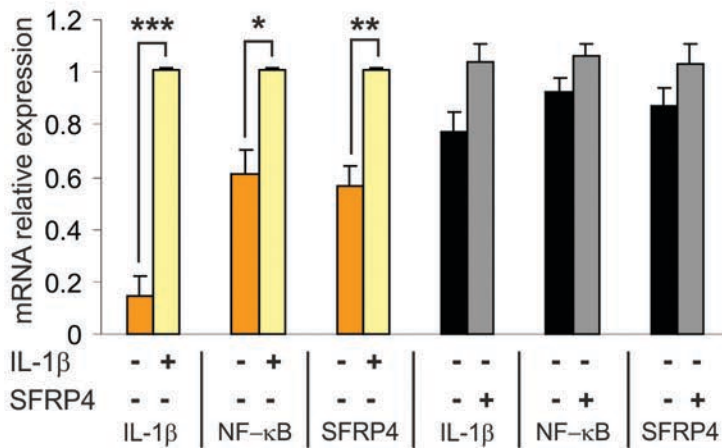
(G) Study subjects were categorized into quartiles based on serum SFRP4 at visit 1, and the histogram shows the number of non-diabetic individuals and converters, respectively, in different quartiles. N=30, 31, 31, and 31 individuals in each quartile.

(H) Kaplan-Meier plot which shows the cumulative increase of individuals diagnosed with T2D over a period of up to 15 years in different quartiles of serum SFRP4 at visit 1.

Data are mean $\pm$ s.e.m. *p<0.05; **p<0.01.

A**B****C****D****E****F****G**



A**B****C****D****E****F****G****H**