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Increased expression of the diabetes gene *SOX4* reduces insulin secretion by impaired fusion pore expansion

Stephan C. Collins^{1,2,*†}, Hyun Woong Do^{1,*}, Benoit Hastoy^{1,*}, Alison Hugill^{3,*}, Julie Adam¹, Margarita V. Chibalina¹, Juris Galvanovskis^{1,4}, Mahdieh Godazgar¹, Sheena Lee⁴, Michelle Goldsworthy³, Albert Salehi^{5,6}, Andrei I. Tarasov^{1,7}, Anders H. Rosengren⁵, Roger Cox³, and Patrik Rorsman^{1,6,7}

¹OCDEM, Radcliffe Department of Medicine, Oxford OX3 7LJ, UK

²Université de Bourgogne Franche-comté 9E Boulevard Jeanne d'Arc 21000 France

³MRC Harwell, Mammalian Genetics Unit, Harwell Campus, OX11 0RD, UK

⁴Henry Wellcome Centre for Gene Function, Department of Physiology, Anatomy, and Genetics, University of Oxford, Parks Road, Oxford, Oxon OX1 3PT, UK

⁵Lund University Diabetes Centre, Department of Clinical Sciences, Skåne University Hospital Malmö, Lund University, 20502 Malmö, Sweden

⁶Department of Neuroscience and Physiology, University of Göteborg, Box 430, 40530, Göteborg, Sweden

⁷Oxford National Institute of Health Research, Biomedical Research Centre, Churchill Hospital, Oxford OX3 7LJ, UK

Abstract

The transcription factor Sox4 has been proposed to underlie the increased type-2 diabetes risk linked to an intronic SNP in *CDKALI*. In a mouse model expressing a mutant form of Sox4, glucose-induced insulin secretion is reduced by 40% despite normal intracellular Ca²⁺ signalling and depolarization-evoked exocytosis. This paradox is explained by a 4-fold increase in kiss-and-run exocytosis (as determined by single-granule exocytosis measurements), in which the fusion pore connecting the granule lumen to the exterior only expands to a diameter of 2 nm that does not allow the exit of insulin. Microarray analysis indicated that this correlated with an increased expression of the exocytosis-regulating protein Stxbp6. In a large collection of human islet preparations (n=63), *STXBP6* expression and GIIS correlated positively and negatively with *SOX4* expression, respectively. Overexpression of *SOX4* in the human insulin-secreting cell

Corresponding author: Stephan C. Collins, collinss@igbmc.fr.

*Co-first author

†Current address: Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France

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EndoC- β H2 interfered with granule emptying and inhibited hormone release, the latter effect was reversed by silencing of *STXBP6*. These data suggest that increased *SOX4* expression inhibits insulin secretion and increased diabetes risk by upregulation of *STXBP6* and an increase in kiss-and-run exocytosis at the expense of full fusion. We propose that pharmacological interventions promoting fusion pore expansion may be effective in diabetes therapy.

Keywords

type-2 diabetes; insulin; exocytosis; *Sox4*; *Stxbp6*

Introduction

Reduced glucose-induced insulin secretion (GIIS) is a hallmark of type-2 diabetes (1). This disease results from a complex crosstalk between lifestyle factors (like body weight and age) and genetics (1). Insulin is released by Ca^{2+} -dependent exocytosis of insulin-containing secretory granules. Exocytosis involves the fusion of the granular membrane with the plasma membrane, a process that is initiated by the establishment of a narrow fusion pore connecting the granule lumen with the extracellular space (2). Effective release of insulin requires the rapid expansion of the fusion pore so that the secretory granules integrate with the plasma membrane.

Genome-wide association studies have led to the discovery of >100 loci associated with increased type-2 diabetes risk (3; 4), many of which are believed to act through reduced β -cell mass or interference with insulin secretion (5). For most of these loci, gene annotation is only tentative and based on the proximity of the single nucleotide polymorphism (SNP) to a certain gene (4). An intronic SNP in the *CDKAL1* gene (rs7756992) is associated with a 50% increase in the risk of type-2 diabetes (6–9). However, recent data indicate *CDKAL1* is not the causative gene (9) and that its effect is instead mediated by altered expression of a nearby gene encoding the transcription factor *SOX4* (10).

Here we show that increased *SOX4* expression is associated with reduced GIIS and elevated plasma glucose and that increased *SOX4* expression impedes the delivery of insulin into the extracellular space via increased expression of the exocytosis-regulating protein *STXBP6* (amisyn). Overexpression of amisyn promotes the stabilization of the fusion pore and locks it in a partially expanded state (2–3 nm) (11). This prevents the exit of insulin from the granule lumen into the extracellular space and thus impairs insulin secretion.

Research design and Methods

Animal husbandry

Animal work was approved by the local and national authorities. Mice used were described previously (12) but are heterozygous for the *Sox4* mutation and were *Insr*^{+/+}. Mice were killed by cervical dislocation and islets isolated by liberase digestion and handpicking as previously described (13). Static and dynamic measurements of insulin secretion were performed as in (12). Insulin was measured using a mouse insulin ELISA kit (Millipore, Hertfordshire, UK).

Mouse microarray studies

Islets were isolated from 22-week old male wild-type and mutant mice (four animals per genotype) and RNA extracted using a RNeasy mini-kit (Qiagen) and validated using an Agilent 2100 bioanalyzer (Agilent Technologies). Labeled and fragmented cRNA was hybridized to the Affymetrix 430 2.0 whole mouse genome microarray and processed on an Affymetrix GeneChip Fluidics Station 450 and Scanner 3000 (Affymetrix).

Cell transfections, siRNA and secretion assays

The constructs coding for GFP-tagged mouse and human *Sox4* and *Stxbp6* were purchased from OriGene Technologies (Rockville MD, USA). The Y123C mutation was introduced into mouse *Sox4* using QuikChange protocol (Agilent Technologies). INS-1 832/13 cells were transfected using Lipofectamine RNAiMAX (Life Technologies, Paisley, UK). The following siRNAs were used: ON-TARGETplus siRNA SMARTpool for *Stxbp6* gene and ON-TARGETplus Non-Targeting Control siRNA (Thermo Scientific, Hemel Hempstead, UK). After 24h, the cells were cotransfected with human growth hormone (hGH) and either DsRed or GFP-tagged mouse *Sox4* or *Stxbp6* using GeneJuice (Merck Millipore, Nottingham, UK). Supernatants and cell pellets were collected and the amount of growth hormone was measured by using an hGH ELISA kit (Roche Diagnostics, West Sussex, UK). EndoC- β H2 cells (14) were transfected with Human specific ON-TARGETplus siRNA SMARTpools (Thermo Scientific, Hemel Hempstead, UK) for *STXBP6* and *SOX4* were used in siRNA knockdown experiments. siRNA and transfections were performed as described for INS1-832/13 cells. Quantitative analysis of gene expression was performed using QuantiFast SYBR Green PCR kit and gene specific QuantiTect Primer Assays (both from Qiagen). Expression was calculated using Ct method with GAPDH as a reference gene.

Intracellular calcium measurements

Intracellular calcium concentration ($[Ca^{2+}]_i$) was assessed in freshly isolated intact islets using a dual wavelength PTI system (PTI, Monmouth, USA) fitted on an inverted Zeiss microscope allowing ratiometric measurements using the probe fura-2AM (Invitrogen, Paisley, UK) as described previously (15).

Whole-cell measurements of Ca^{2+} currents and exocytosis

For patch-clamp measurements, islets were dissociated into single β -cells. In whole-cell measurements, insulin-secreting β -cells were identified based on their larger size (>5.5 pF) and complete inactivation of the Na^+ current at -70 mV (see ref. no (16)). Exocytosis and whole-cell Ca^{2+} currents were recorded using an EPC-10 amplifier and the Pulse software (Heka Electronics, Lamprecht/Pfalz, Germany) as described previously (15). Single exocytotic events and fusion pore expansion were detected in the cell-attached configuration. The standard extracellular solution described in (16) supplemented with 20 mM glucose was used as the pipette-filling medium. The PATCHMASTER software (Heka electronics) together with the EPC10 implements an 'internal calibration'. They automatically correct for phase-shifts and frequency-dependent attenuation when a sinusoidal voltage command of 25

kHz is generated (17). The scaled apparent capacitance (Im/ω) and conductance (Re) are then calculated online by the software.

P2X₂ receptor expression and current analysis

P2X₂ currents were recorded in the whole-cell configuration in identified β -cells as described previously (18,19). Experiments in EndoC- β H2 cells were performed after transfecting cells with both *STXBP6* as described above and P2X₂ receptors (as described in (20)). To evoke exocytosis, the cells were intracellular medium contained with 0-9 mM CaCl₂/10 mM EGTA mixture (calculated free [Ca²⁺]_i: 0 or 2 μ M), 3 mM Mg-ATP and 0.1 mM cAMP. The current spikes reflecting vesicular release of ATP were analyzed using the Mini Analysis Program 6.0.3 (Synaptosoft, Decatur, USA) to determine the charge (integrated current; Q) and rise times ($t_{10-90\%}$) and half-widths (HW) of the individual events (20).

Electron microscopy

Isolated islets were washed in PBS and fixed in cold 2.5 % glutaraldehyde in PBS for 1h at 4°C and washed twice in PBS. Islets were incubated for 1h in 1% Osmium tetroxide and washed twice in PBS. Islets were dehydrated through an ethanol series for 5–10min each and then embedded in Agar 100 resin mix (Agar Scientific) pending sectioning and electron microscopy.

Human islet insulin secretion and microarray studies

Islets from cadaveric donors of European ancestry were provided by the Nordic Islet Transplantation Programme (<http://www.nordicislets.org>). All procedures were approved by the local ethics committee at Lund University. The islets were cultured in supplemented CMRL 1066 (ICN Biomedicals) for 1–9 days prior to RNA preparation. Total RNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen). RNA quality and concentration were measured using an Agilent 2100 bioanalyzer (Bio-Rad) and Nanodrop ND-1000 (NanoDrop Technologies). Total RNA was converted into biotin-targeted cRNA, and the biotin-labeled cRNA was fragmented into strands with 35 to 200 nucleotides. This was hybridized onto a Affymetrix GeneChip® Human Gene 1.0 ST chip overnight in a GeneChip® Hybridization 6400 oven. The arrays were washed and stained in a GeneChip® Fluidics Station 450. Scanning was carried out with the GeneChip® Scanner 3000 and image analysis was done with the GeneChip® Operating Software. Data normalization was performed using Robust Multi-array Analysis. Insulin secretion in human islets was measured as described previously (21). Microarray expression data have been deposited at GEO under the accession number GSE50398.

Results

Impaired insulin secretion in *Sox4^{mt}* mice

Mice harboring a point mutation (Y123C) in *Sox4* (*Sox4^{mt}* mice) have previously been reported to be glucose-intolerant and hypoinsulinemic (12) but the precise mechanism by which this mutation results in diabetes is elusive. As shown in Figure 1A-B, islets from *Sox4^{mt}* mice have reduced (-50%) GIIS over a range of glucose concentrations, despite

similar insulin content, compared to wild-type (*Sox4wt*) islets. In dynamic (perfusion) measurements, both *Sox4wt* and *Sox4mt* islets responded to an elevation of glucose with a biphasic stimulation of insulin secretion (Fig. 1C) but GIIS was equally reduced at all times and the secretion defect could not be corrected by the K_{ATP} channel blocker tolbutamide (12).

Glucose-induced $[Ca^{2+}]_i$ increases, Ca^{2+} entry and exocytosis normal in *Sox4mt* β -cells

Insulin secretion is secondary to electrical activity and the associated elevation of cytoplasmic-free Ca^{2+} ($[Ca^{2+}]_i$) (22). We measured β -cell $[Ca^{2+}]_i$ in intact *Sox4wt* and *Sox4mt* islets (Fig. 2A). Both *Sox4wt* and *Sox4mt* islets responded to high glucose with a marked, sustained and reversible increase in $[Ca^{2+}]_i$. Tolbutamide was equally effective at increasing $[Ca^{2+}]_i$ regardless of the genotype.

Since the reduction of insulin secretion in *Sox4mt* islets was neither corrected by tolbutamide, nor correlated with reduced $[Ca^{2+}]_i$, we hypothesized that the mutation instead interferes with insulin exocytosis (i.e. beyond the increase in $[Ca^{2+}]_i$). We thus compared insulin exocytosis in *Sox4wt* and *Sox4mt* β -cells by whole-cell capacitance measurements (23). Exocytosis was elicited by a train of depolarizations roughly emulating the bursts of action potentials that normally trigger insulin secretion (24). Surprisingly, the exocytotic responses were not reduced in *Sox4mt* β -cells (Fig. 2B). The magnitude of the voltage-gated Ca^{2+} currents in *Sox4mt* β -cells was the same as in *wt* cells (Fig. 2C), making it unlikely that an exocytotic defect is obscured by a compensatory increase Ca^{2+} entry.

Subtle effects on granule ultrastructure and number in *Sox4mt* β -cells

Ultrastructurally, *Sox4mt* β -cells appeared normal (Fig. 3A-B) but the granule density was increased (Fig. 3C) whereas the dense core areas and granule diameters were marginally reduced (Fig. 3D-E).

Slow granule emptying in *Sox4mt* β -cells

The secretory granules of pancreatic β -cells store ATP together with insulin (25). We monitored single-granule release of ATP by infecting β -cells with an adenovirus encoding ionotropic purinergic P2X₂ receptors (26) (Fig. 4A-B). Exocytosis was triggered by intracellular application of high $[Ca^{2+}]_i$ (2 μ M). Quantal (single-granule) release of ATP activates the P2X₂ receptors near the release sites and thus gives a rise to transient membrane currents (19). The frequency of the exocytotic events thus detected was not different in *Sox4wt* and *Sox4mt* β -cells (0.24 ± 0.04 and 0.24 ± 0.04 events/s; not shown), in agreement with the whole-cell capacitance measurements of exocytosis (see Fig. 2C). Importantly, many of the current transients recorded in *Sox4mt* β -cells exhibited slower kinetics than those observed in *Sox4wt* cells (Fig. 4C). The size and shape of these transients reflect the amount and speed of ATP release (25). The charge (Q) and kinetics (rise times and half-widths) were measured as illustrated in Supplemental Figure 1. The cumulative histograms of the rise times in *Sox4wt* and *Sox4mt* β -cells are compared in Figure 4D. Whereas 70% of the events in *Sox4wt* β -cells had a rise time of <5 ms, 70% of the events in *Sox4mt* β -cells had rise times >5 ms. The events recorded in *Sox4mt* β -cells were also longer and the distribution of the half-widths was biphasic (Fig. 4E); $\sim 20\%$ of the events had

duration of 50-200 ms (highlighted by arrow). However, no differences in the distribution of the charge (reflecting the total amount of ATP released) were detected (Fig. 4F).

Impaired fusion pore expansion in *Sox4mt* β -cells

We performed on-cell measurements of single-granule exocytosis and fusion pore conductance within the piece of membrane enclosed by the recording electrode. This technique allows fusion pore conductances to be measured for pores up to 10 nm in diameter (27) (Fig. 5A). In both *Sox4wt* and *Sox4mt* β -cells, stepwise increases in membrane capacitance (reflecting exocytosis of single insulin granules) were observed. The average amplitude of these steps was 5.2 ± 0.5 (n=119) and 5.3 ± 0.4 fF (n=119). Assuming spherical geometry and a specific membrane capacitance of $10 \text{ fF} \mu\text{m}^{-2}$, these values correspond to a granule diameter of 0.4 μm , in reasonable agreement with the ultrastructural measurements (Fig. 3E). In *Sox4wt* β -cells, 88% of capacitance steps (Fig. 5B) were either associated with short-lived or no increases at all in membrane conductance (Fig. 5A, left), indicating rapid and irreversible expansion of the fusion pore and complete integration of the granule membrane with the plasmalemma (27). By contrast, in *Sox4mt* β -cells, 48% of capacitance steps often flickered between the baseline and an elevated level, as expected for ‘kiss-and-run’ exocytosis (28). This difference between *Sox4wt* and *Sox4mt* β -cells is statistically significant ($P=8.10^{-7}$ by χ^2 test). During these ‘flickery’ capacitance steps, persistent increases in membrane conductance were observed (Fig. 5A, right). The mean conductance increase averaged 340 ± 31 pS (n=58). Assuming a cylindrical pore length of 15 nm (i.e. 2×7.5 nm; 7.5 nm being the thickness of a single membrane layer) and a solution resistivity of $66.7 \Omega \cdot \text{cm}$ (equation 11.1 in (29)), this value corresponds to a fusion pore diameter of 1.8 ± 0.2 nm (30). The cumulative distribution of the estimated fusion pore diameter is shown in Fig. 5C. In wild-type β -cells, the average fusion pore conductance and diameter were estimated to be 335 ± 42 pS and 1.6 ± 0.3 nm (n=14). It has been reported that the likelihood of kiss-and-run exocytosis correlates with granule diameter (29). We examined this aspect in *Sox4mt* β -cells. We found that capacitance increase associated with kiss-and-run exocytosis and full fusion averaged 6.3 ± 0.7 (n=57) and 4.5 ± 0.4 fF (n=62; $P < 0.01$), respectively. This is opposite to what is expected from Laplace’s law and argues that factors other than the diameter determines whether a granule undergoes kiss-and-run or full fusion exocytosis.

Gene expression microarrays

To identify target genes of *Sox4* in β -cells, we performed a gene expression microarray on *Sox4wt* and *Sox4mt* islets (Supplemental Table 2). A total of 60 genes were up-regulated (1.5- to 2.9-fold) and 56 genes were down-regulated (1.5- to 3.3-fold). Given the phenotype, genes involved in exocytosis are particularly interesting. These include *Stxbp6* (also known as amisyn; up-regulated 2.1-fold), *Doc2b* (down-regulated 1.8-fold) and *Rab3c* (down-regulated 2-fold). Total ablation of *Doc2b* was recently found to have only a small (10%) inhibitory effect on GIIS (30). Expression of a GTPase-deficient mutant of *Rab3c* has a very moderate effect on insulin secretion (31). Reduced expression of *Doc2b* and *Rab3c* are therefore unlikely to explain the reduction of GIIS in *Sox4mt* islets. However, overexpression of *Stxbp6* has been reported to slow the expansion of the fusion pore in adrenal chromaffin cells (11). The latter effect is obviously reminiscent of that we observe in *Sox4mt* β -cells.

Overexpression of *Sox4mt* interferes with glucose-induced secretion in rat insulinoma cells

In islets from *Sox4mt* mice, *Sox4* expression is increased by 100% compared to wild-type islets (Supplemental Fig. 3 in (12)). We therefore hypothesised that the effects on GIIS in *Sox4mt* islets may be mediated via increased Sox4 activity which, in turn, leads to increased *Stxbp6* expression and impaired fusion pore expansion. We compared the effects of similarly overexpressing *Sox4wt* and *Sox4mt* on *Stxbp6* expression in INS-1 832/13 cells (Supplemental Fig. 2). Both *Sox4wt* and *Sox4mt* increased *Stxbp6* expression, but the effect was stronger for the mutant (Fig. 6A). In secretion experiments, down- and up-regulation of *Stxbp6* stimulated and inhibited glucose-induced growth hormone release (used as a proxy for insulin secretion in transfected cells (32)) by 30% and 40%, respectively (Fig. 6B). Whilst overexpression of *Sox4wt* had only a small inhibitory effect (-15%) on hormone release, stronger inhibition (-40%) was observed when overexpressing *Sox4mt* (Fig. 6B). Silencing *Stxbp6* largely reversed the inhibitory effect of overexpressing *Sox4mt*.

Increased SOX4 expression in human islets correlates with reduced GIIS

To extend these data to man, we correlated *SOX4* expression to donor HbA1c levels (a surrogate measure of long-term plasma glucose control), *STXBP6* expression and GIIS in a large collection of human islet preparations. There was a positive association between *SOX4* expression and HbA1c levels (Fig. 7A) and *STXBP6* expression (Fig. 7B) but a negative correlation between *SOX4* and GIIS (Fig. 7C). In agreement with published data (33), there was a tendency towards higher *SOX4* expression in islets from donors with type-2 diabetes (defined as established disease or HbA1c>6%; Supplemental Fig. 3A). There was also a positive association between *CDKAL1* and *SOX4* expression (Supplemental Fig. 3B).

These analyses suggest that increased *SOX4* expression, via elevated *STXBP6*, reduces GIIS in human β -cells. We confirmed this by experimentally up- and down-regulating *SOX4* and *STXBP6* in the glucose-responsive (Supplemental Fig. 3C) human β -cell line EndoC- β H2 (14). Moderate overexpression (+150%) of *SOX4* in EndoC- β H2 cells was associated with a 250% increase in *STXBP6* expression (Fig. 7D), an effect that was fully antagonised by knockdown of *STXBP6* by *siRNA* (*si-STXBP6*). By contrast, silencing of *SOX4* did not reduce *STXBP6* expression below the low basal level. Overexpression of *SOX4* inhibited insulin secretion by 50%, an effect that was reversed by knockdown of *STXBP6* (Fig. 7E). Overexpression of *SOX4* also increased expression of syntaxin-1a (*STX1A*; Supplemental Fig. 3D), possibly to compensate for the binding of stxbp6 to syntaxin-1 (34), but this effect is unlikely to explain the suppression of insulin secretion as a positive correlation between *STX1A* expression and GIIS has been reported (35). Down-regulation of *SOX4* neither affected *STXBP6* expression (Fig. 7D), nor hormone release (Fig. 7F).

To examine the effect of increased *STXBP6* expression on granule emptying in human insulin-secreting cells, we expressed P2X₂Rs in EndoC- β H2 cells and monitored single-granule ATP release (Fig. 7G). Overexpression of *STXBP6* resulted in a marked decrease in ATP release (measured as a decrease in *Q*, Fig. 7H). The mean charge of events was reduced by 90%. However, the frequency of the events was increased from 1.04±0.38 to 2.8±1.3 Hz (n=9 or 10; not statistically different). Combining the two effects suggest that there is a 70%

reduction in stimulated ATP release following overexpression of *STXBP6* (Fig. 7I), in reasonable agreement with the 60% suppression of stimulated hGH release (Fig. 6B). Similar results were obtained in INS 832/13 cells. No ATP release events were observed when the EndoC- β H2 cells were infused with Ca^{2+} -free medium (Fig. 7F, top trace)

Discussion

Type-2 diabetes represents an epitome of a polygenic disorder (1). Genome-wide association studies have led to the identification of ~120 common gene variants (SNPs) with increased type 2 diabetes risk (36). For the majority of these gene variants, the precise cellular/molecular mechanisms underlying the increased disease risk remain obscure. The diabetes-associated SNP rs7756992 is commonly referred to *CDKAL1* but recently it was suggested that this locus increases disease risk via *SOX4* rather than *CDKAL1* (see (10)). Intriguingly, its expression is increased in diabetic islets (35) but whether increased expression of *SOX4* is causally linked to type 2 diabetes and (if so) the underlying cell biological mechanism have not been studied.

Here we show that increased *SOX4* expression in human pancreatic islets correlates with reduced GIIS *in vitro* and increased HbA1C levels *in vivo*. Interestingly, we also found a correlation between *CDKAL1* and *SOX4* expression, in keeping with the idea that the *CDKAL1* locus influences the expression of both *CDKAL1* and *SOX4* (10). We also observed a tendency towards increased *SOX4* expression in islets from diabetic donors (Supplemental Fig. 3A), in agreement with what was previously reported based on microarray analysis of a β -cell-enriched fraction obtained by laser capture microdissection (35).

To address the mechanisms by which *SOX4* may interfere with insulin secretion in man, we made use of the *Sox4mt* mouse model, in which islet *Sox4* expression is twice that of wild-type islet (12). We found that GIIS was reduced by ~50% in *Sox4mt* islets, a defect that was not corrected by the sulfonylurea tolbutamide. The reduction of insulin secretion could not be attributed to any impairment of key functional parameters such as glucose-induced $[\text{Ca}^{2+}]_i$, Ca^{2+} entry or exocytosis (determined as depolarization-evoked increases in whole-cell membrane capacitance).

The delivery of insulin and other granule constituents, like ATP (25), into the extracellular space requires the establishment and expansion of a 'fusion pore' connecting the granule lumen and the exterior (27). Normally, the initial opening of the fusion pore is followed by its rapid expansion and, following a short delay, the entire granule 'collapses' into the plasma membrane, ensuring efficient delivery of the high molecular-weight cargo into the extracellular space ('full fusion'). Occasionally, however, fusion pore expansion is halted following the initial opening and may eventually close ('kiss-and-run' exocytosis (27)).

Using on-cell capacitance measurements of individual exocytotic events and the associated fusion pore openings, we found that full fusion was the predominant (~90%) form of exocytosis in *wt* β -cells, in agreement with previous reports (29). However, fusion pore expansion is impaired in *Sox4mt* β -cells and the fraction kiss-and-run release events

increased to ~50% at the expense of full fusion. During the kiss-and-run events, the fusion pore is locked in a partially expanded state with a diameter of 1-2 nm.

We determined the functional impact of this on the evacuation of the granule lumen by measurements of ATP release. The molecular dimensions of ATP are 1.6*1.1*0.5 nm. With the observed distribution of fusion pore diameters, we estimate that ~20% of the total number of release events in *Sox4mt* β -cells (i.e. 43% of the 50% showing persistent fusion pore) have fusion pore diameters smaller/comparable to the narrowest cross-section of ATP (indicated by a vertical dashed line in Fig. 5C). Thus, the fusion pore may function as a molecular sieve that restricts the exit of ATP by steric hindrance. This likely accounts for the slower rise times of ATP release and the component of ATP release events with a duration >20ms (Fig. 4D). Larger molecules like insulin are likely to face an even greater problem exiting via the narrow fusion pore. At concentrations >2 mM, insulin exists as hexamer with a hydrodynamic radius of 5.6 nm (37). Given that the intra-granular insulin concentration is ~100 mM (22), we conclude that insulin is trapped within the secretory granules undergoing kiss-and-run exocytosis. Indeed, the observed decrease in 'full fusion' from 88% to 52% is sufficient to account for the observed 40% reduction of GIIS in *Sox4mt* islets. It is tempting to speculate that the reduction in full fusion accounts for the increased granule density observed in *Sox4mt* β -cells. Although most fusion pores are mostly too small to allow exit of insulin, 10% have a diameter >6 nm. A fusion pore diameter as large as this is sufficient to allow the exit of a hexameric insulin, which might explain the slight reduction in dense core area of the insulin granules (Fig. 3D).

Being a transcription factor, it is not surprising that the expression of >100 genes were affected in *Sox4mt* islets. However, guided by the functional data implicating specifically kiss-and-run exocytosis, we identified *Stxbp6* (amisyn) as a likely mediator. Overexpression of *Stxbp6/STXBP6* mimics the effects of *Sox4/SOX4* on secretion in rat/human insulinoma cells. Conversely, silencing *Stxbp6/STXBP6* reverses the inhibitory effect of overexpressing *Sox4/SOX4*. Unexpectedly, and unlike what has previously been observed in rodent insulinoma cells (12), silencing *SOX4* in human insulinoma cells did not affect hormone release. The mechanism underlying this discrepancy remains unclear.

Our data suggest that increased expression/activity of *SOX4*, via increased expression of *STXBP6* and impaired expansion of the fusion pore, plays a role in insulin secretion and diabetes etiology. This scenario is in agreement with the proposal that *Stxbp6* forms nonfusogenic complexes with syntaxin and thereby contributes to the regulation of SNARE complex formation (35). This is the first example of a major disease that can be linked to defective fusion pore expansion. It is possible that the mechanisms we have uncovered here may become activated during long-term hyperglycaemia, a condition reported to be associated with an increase in kiss-and-run exocytosis (38). It appears that the effect of increased *STXBP6* expression is graded. Whereas a moderate increase (up to +100%) results in an increased occurrence of kiss-and-run exocytosis (as seen in the *Sox4mt* mice), stronger overexpression may result in exocytosis being aborted before granule emptying (see Schematic in Supplemental Fig. 4). In cells overexpressing *STXBP6*, we observed small and short events, possibly reflecting repetitive openings/closures of the fusion pore releasing a puff of ATP every time.

If increased *SOX4* expression is part of the disease etiology and if it acts by interference with fusion pore expansion, then it may seem paradoxical that type-2 diabetes is not also associated with defects of neurotransmission. There is in fact a link between type-2 diabetes and neuropsychiatric disorders (39). It should be noted, however, that for low molecular-weight neurotransmitters, slowed/partial fusion pore expansion will only have a marginal effect on their release because they are small enough to pass through a constricted fusion pore.

This study illustrates how detailed cell physiological studies can help us move from SNPs, via identification of the actual gene involved, to a fuller understanding of the causal mechanisms. These findings raise the interesting possibility that pharmacological procedures promoting full fusion may correct the insulin secretion defect in type 2 diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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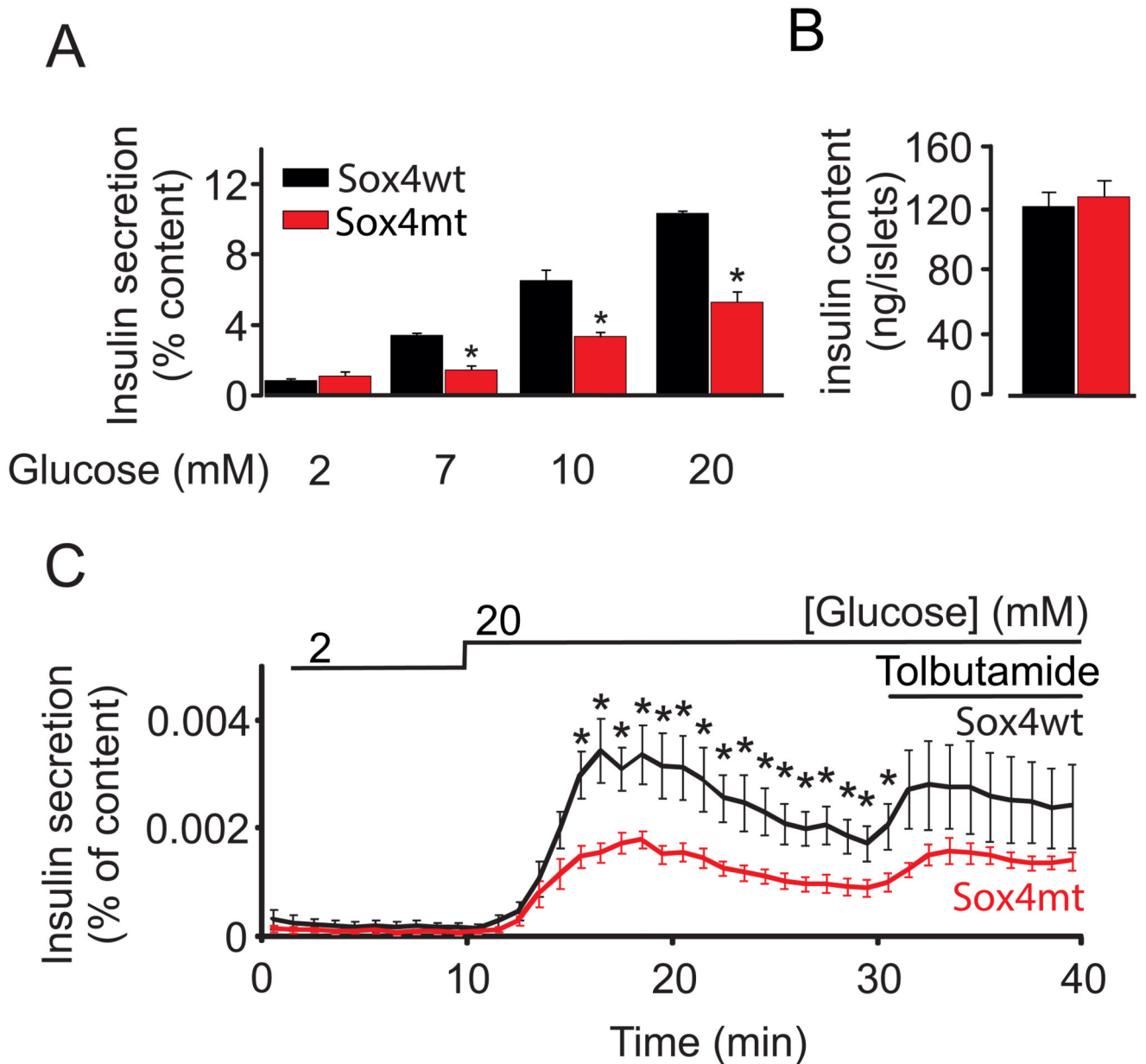


Figure 1. Impaired insulin secretion in *Sox4mt* islets. *A-B*: Insulin secretion (*A*) and content (*B*) measured in intact islets in static incubations. Islets were exposed to increasing glucose concentrations as indicated. Data are from 3 animals per genotype (with 3 technical replicates of 5 islets per animal) experiments. * $P < 0.05$. *C*: Dynamic measurements (perfusion) in *Sox4mt* (red) and *Sox4wt* (black) islets. Data are from 6 animals per genotype with 50 islets per animal and normalized to insulin content * $P < 0.05$ using a one-way Anova, repeated measures.

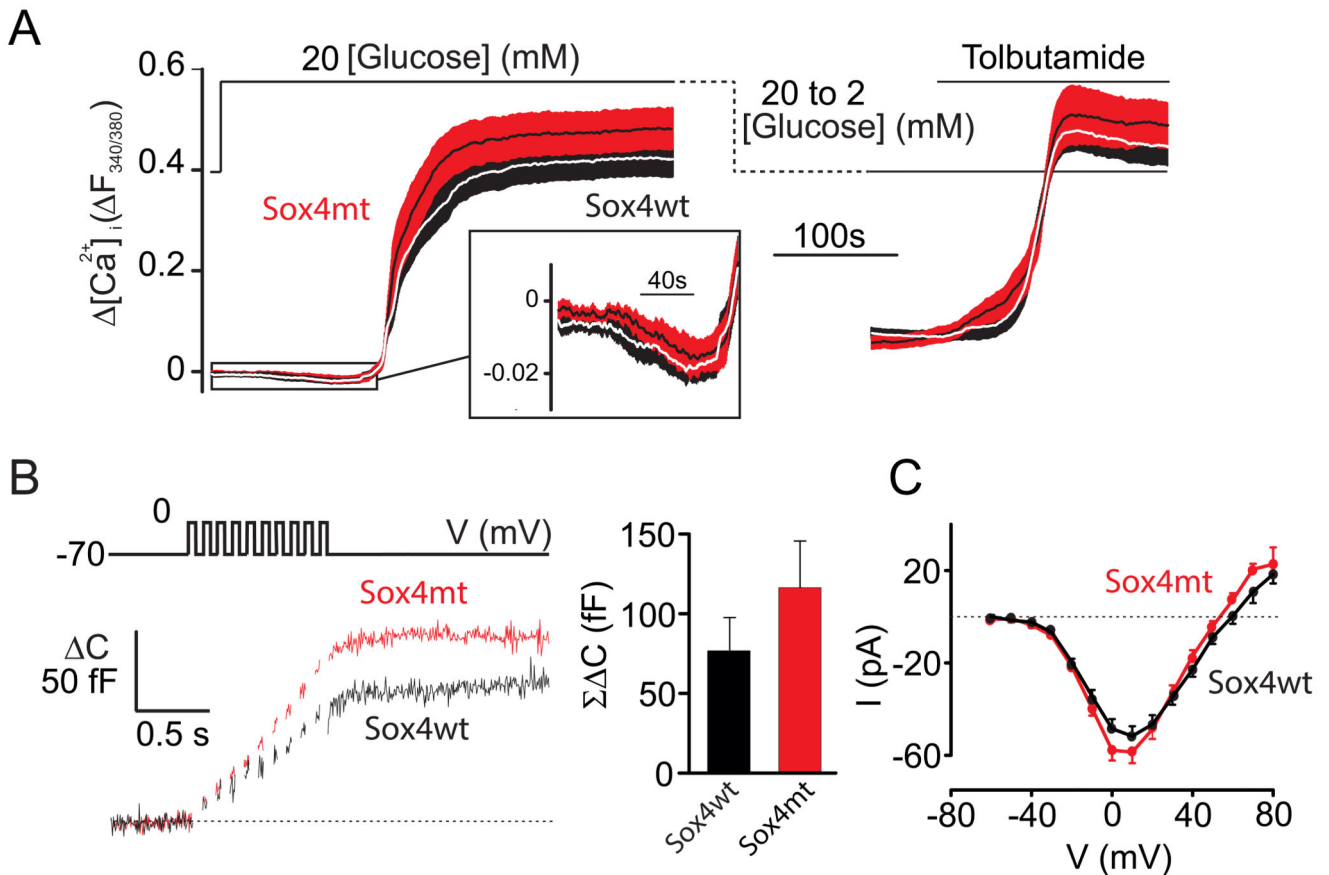


Figure 2.

No impairment of $[Ca^{2+}]_i$ signaling, exocytosis (measured via whole cell capacitance) and Ca^{2+} currents. **A:** Glucose- and tolbutamide-induced increases in cytosolic Ca^{2+} in *Sox4wt* (black) or *Sox4mt* (red) islets. The glucose concentration was varied as indicated above the traces. Tolbutamide was added at a concentration of 0.2 mM. Traces are mean \pm S.E.M. of 9 experiments for each genotype (3 islets per animal and 3 animals per genotype) and have been baseline corrected to correct for fading of the fluorophore. **B:** Increase in membrane capacitance (ΔC) during a train of ten 50 ms depolarizations from -70 mV to 0 mV (V) in *Sox4wt* (black) and *Sox4mt* (red) β -cells. Histogram (below) compares the total increase in membrane capacitance ($\Sigma \Delta C$) evoked by the train of depolarizations in *Sox4wt* (black) and *Sox4mt* (red) β -cells (n=24-28 β -cells from 4 mice per genotype). **C:** Peak current (I)-voltage (V) relationship for voltage-gated Ca^{2+} currents recorded from single *Sox4wt* (black) and *Sox4mt* (red) β -cells during depolarizations from -70 mV to membrane potentials between -60 and +80 mV (n=13-16 β -cells from 4 mice per genotype). The horizontal dotted line indicates zero current level.

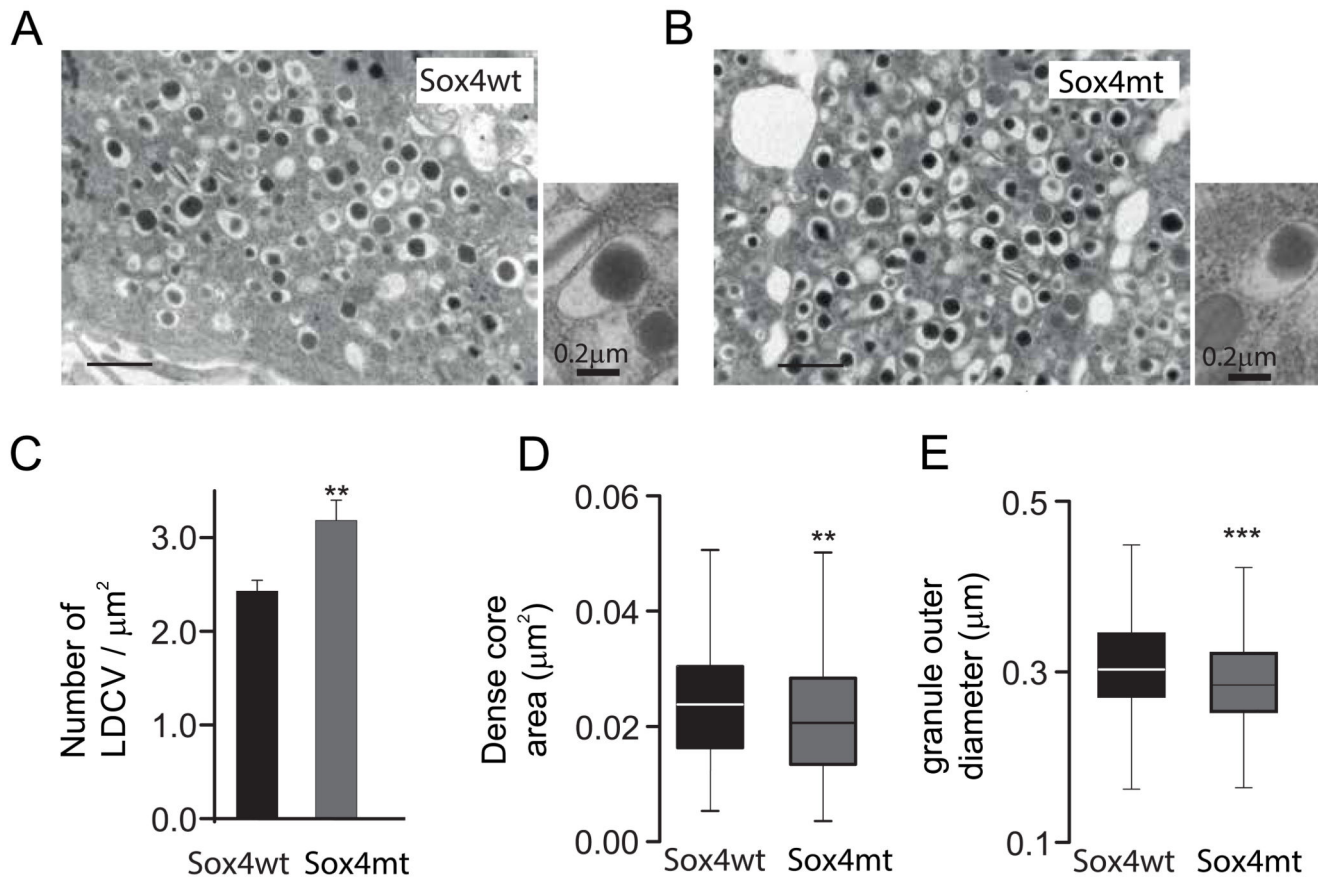


Figure 3.

Subtle effects on cellular ultrastructure in *Sox4mt* β -cells. *A-B*: Representative electron micrographs from *Sox4wt* (*A*) and *Sox4mt* (*B*) β -cells. Scale bars: $1\mu\text{m}$. *C-E*: Granule density (*C*) and box plots, depicting groups of data according to their quartiles, of the dense core area (*D*) and granule diameter (*E*) in *Sox4wt* (black) and *Sox4mt* β -cells (grey). At least 6 β -cells from 2 animals per genotype were analyzed. Data in (*D*) are mean \pm S.E.M. ** $P < 0.01$. Distributions of core areas and granule diameters were estimated by counting >382 granules per genotype. ** $P < 0.005$; *** $P < 0.0005$ (by Mann-Whitney U-test).

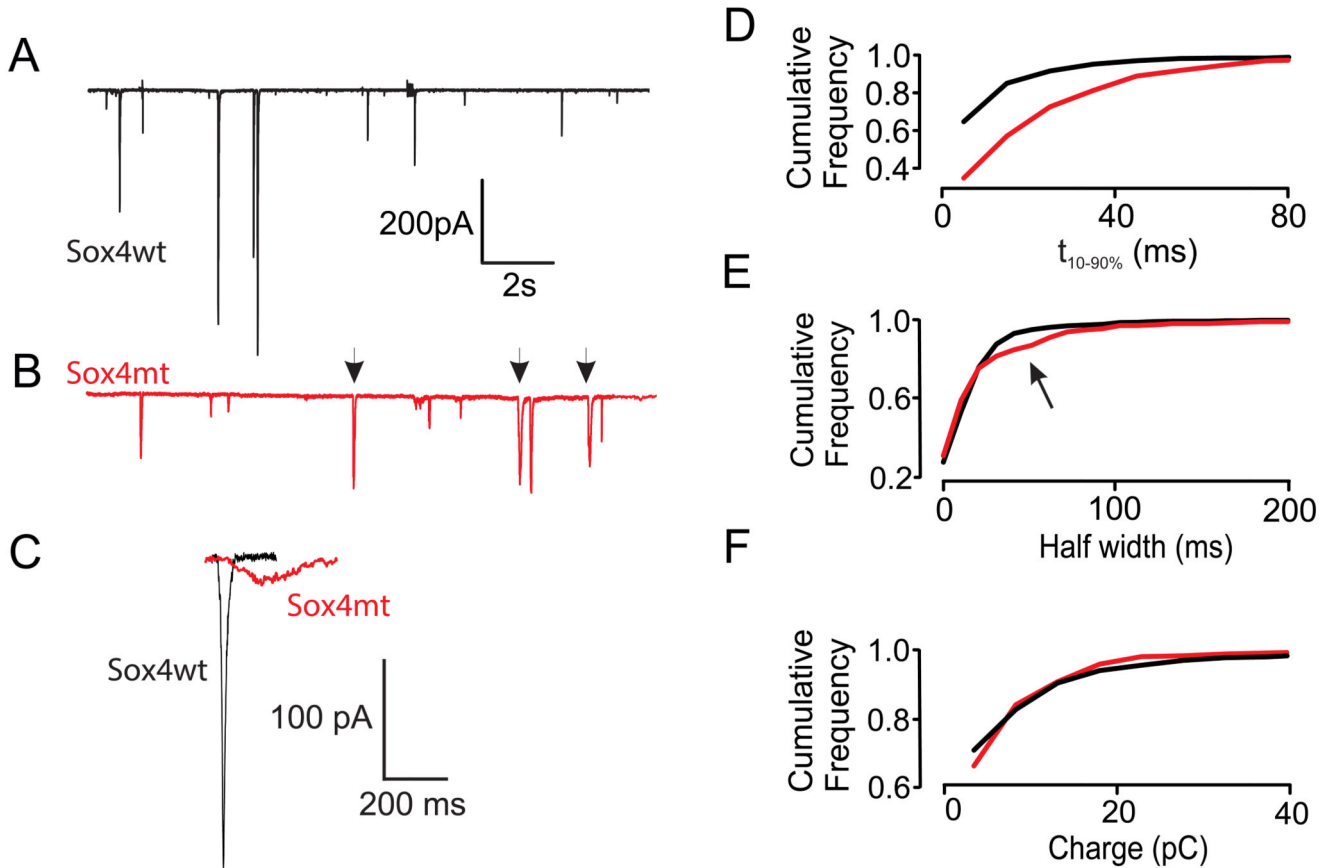


Figure 4.

Delayed granule emptying in *Sox4mt* β -cells. *A*: Release of ATP monitored in individual *Sox4wt* β -cells expressing ionotropic P2X₂ receptors infused with 2 μ M free [Ca²⁺]_i. *B*: As in (*A*) but using from *Sox4mt* β -cells. Arrows indicate slow ('kiss-and-run') events. *C*: Rapid event recorded in a *Sox4wt* β -cell (black) and slow 'kiss-and-run' events recorded in a *Sox4mt* (red) β -cell. *D*: Cumulative histogram for the rise times in *Sox4wt* (black) and *Sox4mt* (red) β -cells. ($P < 0.005$ by independent-samples Kolmogorov-Smirnov test). *E*: As in (*D*) for half-widths. The arrow indicates that ~20% of release events are of longer duration ($P < 0.005$ by independent-samples Kolmogorov-Smirnov test). *F*: As in (*D-E*) for total charge (Q). Data shown in (*D-F*) based on >368 events obtained from 10 (*Sox4mt*) and 14 (*Sox4wt*) β -cells from 2 mice of each genotype. See also Supplemental Figure 1.

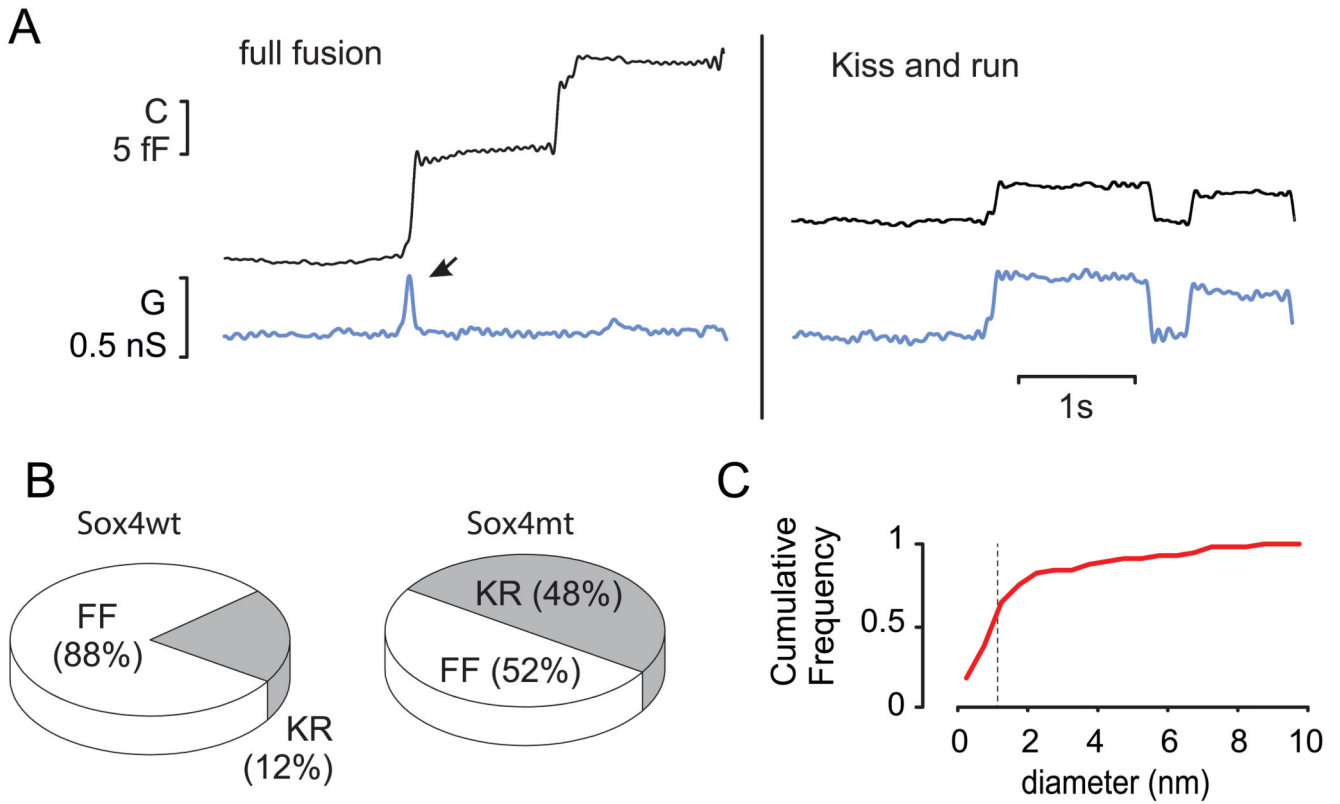
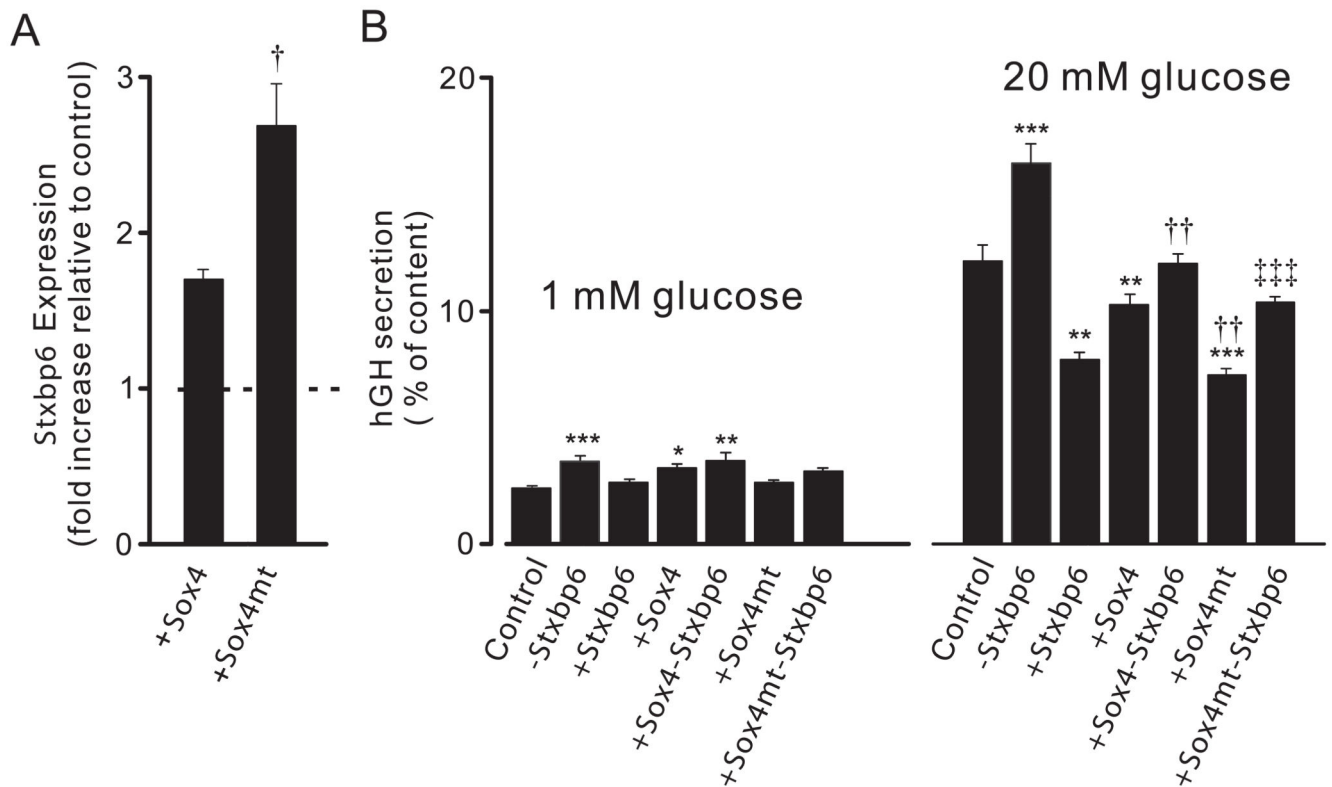
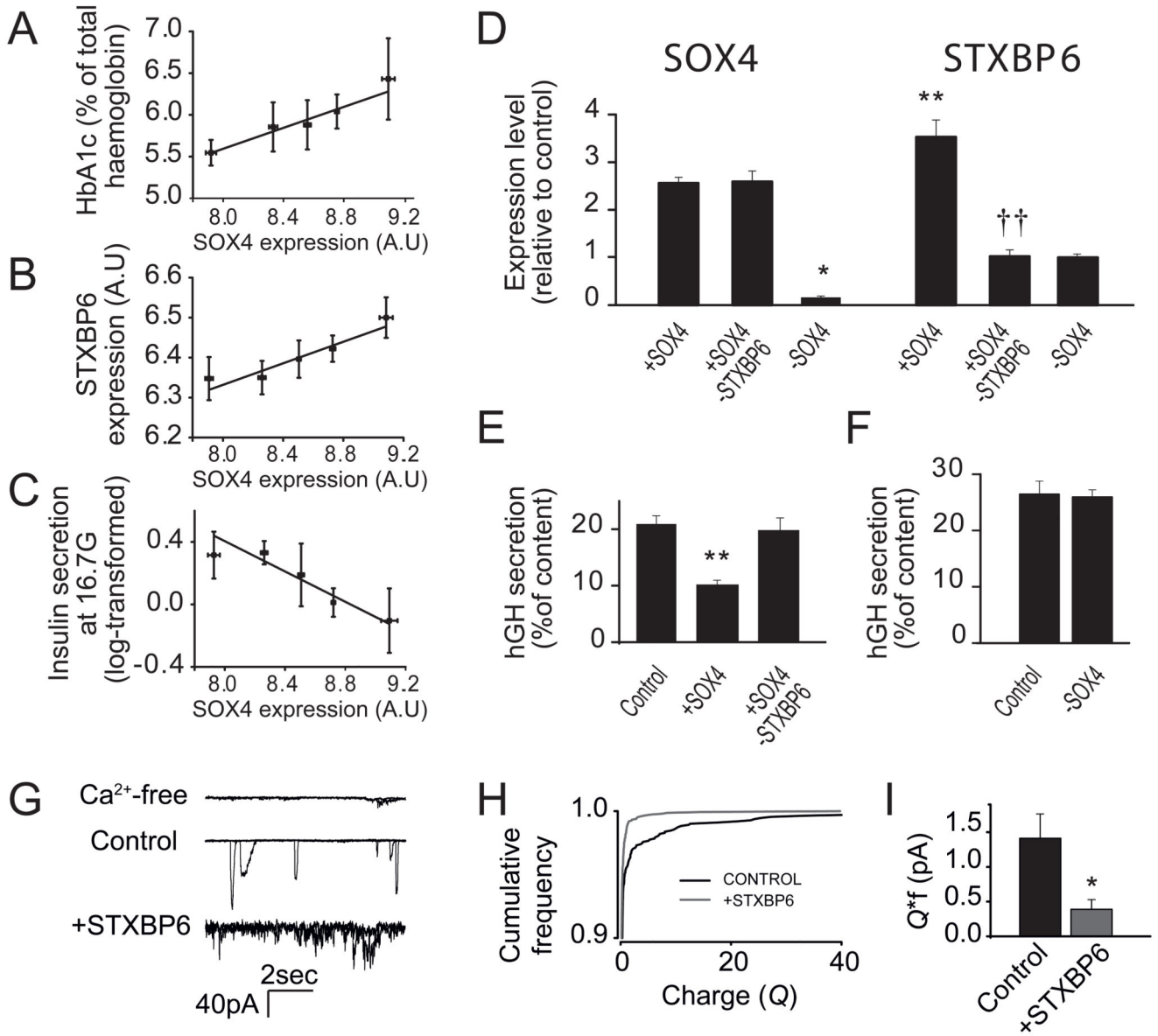


Figure 5.

Impaired fusion pore expansion in *Sox4mt* β -cells. *A*: On-cell measurements of membrane capacitance (*C*) and conductance (*G*) showing two examples of full fusion (FF) from a *Sox4wt* β -cell (left) where the first event is associated with a prominent but short-lived increase in conductance (arrow) and an example of transient exocytosis (kiss-and-run; abbreviated KR) recorded from a *Sox4mt* β -cell (right) where *C* returns to baseline and the capacitance step is associated with a persistent increase in *G*. *B*: Pie charts summarizing the fraction of full fusion or kiss-and-run exocytosis in *Sox4wt* ($n=119$ events in 23 cells from 5 mice) and *Sox4mt* (120 events in 30 cells from 5 mice) β -cells ($P=8.10^{-7}$ by χ^2 test). *C*: Cumulative histogram of the distribution of estimated fusion pore diameters in *Sox4mt* β -cells. The dashed vertical line indicates a fusion pore diameter of 1.1 nm (enough to allow the exit of ATP). Also note that 10% of the events have an estimated pore diameter >6 nm. See also Supplemental Table 1.

**Figure 6.**

Mutant *Sox4* mediates its effects on insulin secretion via *Stxbp6*. *A*: qRT PCR analysis of *Stxbp6* expression in INS-1 832/13 cells transfected with scrambled siRNA + DsRed + hGH (dashed horizontal line; control), scrambled siRNA + *Sox4wt* + hGH (labeled '+*Sox4*') or scrambled siRNA + *Sox4mt* + hGH ('+*Sox4mt*'). † $P < 0.05$ wt vs *Sox4*. *B*: hGH release (as a proxy for insulin release in transfected cells) from INS-1 832/13 cells transfected with scrambled siRNA + DsRed + hGH (labeled 'control'), *si-Stxbp6* + DsRed + hGH ('-*Stxbp6*') and scrambled siRNA + *Stxbp6* + hGH ('+*Stxbp6*'), scrambled siRNA + *Sox4wt* + hGH ('+*Sox4*'), *si-Stxbp6* + *Sox4wt* + hGH ('+*Sox4-Stxbp6*'), scrambled siRNA + *Sox4mt* + hGH ('+*Sox4mt*') and *si-Stxbp6* + *Sox4mt* + hGH ('+*Sox4mt-Stxbp6*') incubated with 1 or 20mM glucose (as indicated). Data are presented as mean \pm S.E.M. of 4 experiments, each with 3 replicates. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ for comparison vs control (1 or 20 mM glucose alone) and †† $P < 0.01$ vs *Sox4wt* and †††† $P < 0.01$ vs *Sox4mt* (by ANOVA and Tukey's tests). See also Supplemental Figure 2.

**Figure 7.**

Effects of *SOX4* and *STXBP6* on human β -cell function. *A*: Relationship between *SOX4* expression and HbA1c levels. $R=0.305$; $P=0.036$; $n=52$. *B*: Relationship between *SOX4* expression and *STXBP6* expression $R=0.321$; $P=0.021$; $n=63$. *C*: Relationship between *SOX4* expression and GIIS. $R=-0.292$; $P=0.043$; $n=48$. For display, data in (*A-C*) have been grouped in quintiles. Best-fit black lines represent Pearson correlation analyses to the individual data points. *D*: qRT PCR analysis of *SOX4* and *STXBP6* expression in human cells transfected with scrambled siRNA + DsRed + hGH (dashed horizontal line; labelled 'Control'), scrambled siRNA + *SOX4**wt* + hGH ('+*SOX4*'), *si-STXBP6* + *SOX4**wt* + hGH ('+*SOX4* -*STXBP6*') and *si-SOX4* + DsRed + hGH ('-*SOX4*'). Data are presented as mean \pm S.E.M. of 3 experiments, each with 3 replicates; ** $P<0.01$ vs control (dashed horizontal line); †† $P<0.01$ vs '+*SOX4*' (by ANOVA and Tukey's tests). *E-F*: As in (*D*) but hGH release

(as a proxy for insulin release in transfected cells) was measured in EndoC- β H2 cells exposed to 20mM glucose + 70mM $[K^+]_o$. ** $P < 0.01$ vs 'control'; †† $P < 0.01$ vs '+*SOX4*' (by ANOVA and Tukey's tests). Mean \pm S.E.M. of 4 experiments, each with 3 replicates. Note that (E) and (F) were carried out independently, and for (F) data are presented as mean \pm S.E.M. of 3 experiments, each with 3 replicates. See also Supplemental Figure 3A-D. *G*: Effects of overexpressing STXBP6 on ATP release in the human insulin-secreting cell line EndoC- β H2. Traces are labeled '*Control*' (cells expressing DsRed alone) and '+*STXBP6*' (cells overexpressing STXBP6). The top trace shows recordings when the cells were infused with Ca^{2+} -free medium (several sweeps have been superimposed). *H*: Cumulative histogram for the charge (Q) of the events in '*Control*' (black) and '+*STXBP6*' cells (red) ($P < 0.005$ by independent-samples Kolmogorov-Smirnov test). Data based on 1100-1400 events in 9-10 cells. *I*: Product of mean charge and frequency of events in '*Control*' and '+*STXBP6*' cells. $P < 0.05$ vs. '*Control*'. See also Supplemental Fig. 4.