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Mapping of *INS* promoter interactions reveals its role in long-range regulation of *SYT8* transcription

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Abstract

Insulin (INS) synthesis and secretion from pancreatic β cells are tightly regulated; their deregulation causes diabetes. Here we map *INS*-associated loci in human pancreatic islets by 4C and 3C techniques and show that the *INS* gene physically interacts with the *SYT8* gene, located over 300 kb away. This interaction is elevated by glucose and accompanied by increases in *SYT8* expression. Inactivation of the *INS* promoter by promoter-targeting siRNA reduces *SYT8* gene expression. *SYT8-INS* interaction and *SYT8* transcription are attenuated by *CTCF* depletion. Furthermore, *SYT8* knockdown decreases insulin secretion in islets. These results reveal a non-redundant role for *SYT8* in insulin secretion and indicate that the *INS* promoter acts from a distance to stimulate *SYT8* transcription. This suggests a function for the *INS* promoter in coordinating insulin transcription and secretion through long-range regulation of *SYT8* expression in human islets.

Keywords

Insulin; chromatin; SYT8; diabetes

INTRODUCTION

The human genome is organized within the nucleus into a compact chromatin structure that facilitates many interactions between what are otherwise distal sites[1]. A few of these long-range interactions have been shown to impact gene transcription in human cells[2–5]. However, the function of chromatin interactions in the regulation of physiological processes and how environmental signals regulate these dynamic interactions in human tissues remain to be determined. We investigated the *INS* gene locus in this study both for its physiological importance, and as a model to study the role of long-range chromatin interactions in the

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AUTHOR CONTRIBUTIONS

Z.X. and G.F. designed the experiments; Z.X. conducted the experiments; G.W., I.C. and K.Z. performed and analyzed Solexa DNA Sequencing experiments; Z.X. and G.F. wrote the manuscript.

establishment of regulatory networks. Most of our knowledge about insulin (*INS*) gene expression and regulation comes from studies done in rodents, especially the mouse. However, there exist two insulin genes in rodents and only one in human and it has become apparent recently that human pancreatic islets contain unique anatomical and functional features they do not share with rodent islets[6–9]. For these and other reasons we made use of intact human pancreatic islets, in which the *INS* gene is active.

The human *INS* gene, located on chromosome 11p15, is flanked by two clusters of imprinted genes. The DNA sequence and gene organization of this 1-Mb gene-dense region are conserved between mouse and human[10]. Insulin transcription and secretion from pancreatic β cells are tightly regulated in response to glucose[11,12]. Glucose markedly stimulates the human *INS* promoter activity in transfected primary islet cells[13,14], actively promotes open chromatin structure and RNA polymerase II recruitment on the endogenous *INS* promoter, and increases *INS* gene transcription in human islets[11,15,16]. The *INS* gene is transcribed at high levels only in pancreatic β cells[17,18]. The mouse *Ins2* gene and its human ortholog physically interact with the distal *H19* imprinted control region (ICR) and enhancers that are located over 100 kb away[19–21]. The *H19* ICR has been the subject of intense study, and recent 4C (circular chromosome conformation capture) analyses have revealed its long-range interactions with other gene loci, in at least two cases affecting transcription of distant genes[22–24]. Although these data provide evidence for the existence of long-range physical contacts involving the *INS* gene, they are limited to the region near *H19*, in non-human tissues, and in cells not expressing insulin.

In this study, we undertook a series of 3C (chromosome conformation capture) and 4C studies[24,25] in human islets to determine patterns of interaction between the *INS* gene and other sites within the nucleus, and to explore possible connections with *INS* expression. We show that, in human islets, the *INS* promoter physically interacts with and positively regulates transcription of the *SYT8* gene located over 300 kb away, and that this gene is a critical regulator of insulin secretion. These results suggest a regulatory function for the *INS* promoter in coupling insulin transcription with secretion through regulation of *SYT8* gene expression in human islets.

RESULTS

4C-Seq analysis reveals *SYT8–TNNI2* locus association with *INS*

Initial 3C studies confirmed that the *INS* gene and the *H19* ICR interact with each other both in normal human primary fibroblasts (NHPF) and at a higher level in human islets (Supplementary Fig. 1). We explored more generally, using the 4C method[24,25], interactions in human islets between the *INS* gene and other genomic sites. This procedure (4C-Seq, Supplementary Fig. 2a) is done essentially as described[25] except for the last few steps.

We first performed 4C-Seq using a part of the *INS* promoter and gene body as the bait (see Supplementary Fig. 2b). This generated 1.8 million unique sequence reads; 29.6% of them were mapped within the 1-Mb region surrounding the *INS* gene. In islets treated with glucose for one hour, the *INS* gene interacted strongly with loci in this 1-Mb region (Fig. 1a, b). Multiple interacting sites were clustered in this region. The analysis was repeated with

islets from another donor and using the other side of the BglII site as the bait (Supplementary Fig. 2b); similar contact patterns were observed for this 1-Mb region ($P<10^{-90}$) (Supplementary Fig. 3a and Supplementary Notes). Thus, the 4C-Seq method is reproducible (see Supplementary Notes). Because we were focused on interactions within this 1-Mb locus, we did not attempt to comprehensively identify more distant regions on chromosome 11 or on any of other chromosomes (Fig. 1a and Supplementary Notes). Instead, we used the 4C data to provide partial information about significant long range contacts that could then be confirmed and explored by 3C methods.

The *INS*-interacting loci in this 1-Mb region (Fig. 1b) are in a part of the genome that is evolutionarily conserved in DNA sequence and gene organization[10] and contain genes that play roles in diverse cellular processes including exocytosis, cellular proliferation and apoptosis, and cell-cell interactions. In this study we focus on one of these genes, synaptotagmin 8 (*SYT8*)[26,27], and its immediate neighbor, *TNNI2*. Other synaptotagmins have been shown to be Ca^{2+} sensors for neurotransmitter release in neurons[28], and thought to regulate insulin secretion in pancreatic β cells[26,27,29–31]. Syt8 could also be involved in this process because its C_2AB domain peptide markedly decreases Ca^{2+}–induced insulin secretion when introduced into primary rat islet cells[26]. However, earlier studies[27] of Syt8 concluded that it did not act as a calcium sensor in endocrine cells or neurons, and did not participate in the calcium-mediated regulation of evoked exocytosis. Thus, whether *SYT8* plays a role in insulin secretion remains unclear.

Our analysis showed that the *INS* gene physically contacted both the promoter and 3′ downstream region of *SYT8* (Fig. 1c). The second *INS*-interacting site is located less than 2 kb away from the transcription start site of *TNNI2*, the neighboring gene of *SYT8* (see Fig. 1c for the location). The *INS* gene did not interact with the *CTSD* gene or the 3′ downstream region of the *LSP1* gene, two flanking genomic regions both located about 60 kb away from the *SYT8-TNNI2* locus (Fig. 1c); this was confirmed in further 3C experiments (see below). Thus, proximity alone is not sufficient for an interaction. Finally, 4C-Seq analysis showed that the *INS* gene did not interact with any of the other *SYT* gene family members such as *SYT7*, *SYT9* or *SYT13* that are also located on chromosome 11 (Fig. 1a, Supplementary Fig. 3b and data not shown).

3C analysis confirms *SYT8–TNNI2* locus interaction with *INS*

To confirm the 4C results (Fig. 1c), we designed quantitative 3C experiments to detect specifically interactions between the *INS* bait and the region containing the *SYT8* and *TNNI2* genes in islets treated with glucose for 1 h. We focused on interactions of the *INS* gene with eight BglII sites in the region, representing the two sites at the *SYT8* promoter and its 3′ downstream site (or the *TNNI2* promoter), two immediately neighboring sites flanking the *SYT8-TNNI2* locus, three sites covering the *LSP1* gene and the only site within the *CTSD* gene (see Fig. 1c for the location). Quantitative 3C assays confirmed strong contacts between *INS* and the *SYT8-TNNI2* locus in islets from a second donor (Fig. 1d). Weak contact between these sites occurred at 2- to 3-fold lower levels in human primary fibroblasts (Fig. 1d). Similar to the 4C-Seq analysis, this 3C assay also showed that the *INS* gene interacted strongly with the *LSP1* gene but not with its 3′ downstream region or the

CTSD gene in islets (Fig. 1d). Islets from the same donor were also used to generate islet-derived precursor cells (hIPC) that express, weakly if at all, the *INS*[32] or *SYT8* gene (Supplementary Fig. 4a). After islets were dedifferentiated into hIPC, *INS* interactions with the *SYT8-TNNI2* locus were decreased by about 3-fold while the *LSP1-INS* interaction in hIPCs, unlike in fibroblasts, was maintained (Fig. 1d). This confirmed the 4C-Seq results showing that the *SYT8-TNNI2* locus interacts with the *INS* gene in islets and suggested that these intranuclear interactions might be regulated, at least partly, by an islet cell-specific mechanism. We note that although *INS* makes strong contacts with *SYT8-TNNI2*, it does not make contact with the nearby *CTSD* gene (Fig. 1d), which is also active in islets (see below), indicating that the contacts are not generated simply through active transcription.

Glucose stimulates *INS–SYT8* interactions and *SYT8* expression

To determine whether glucose could regulate *INS-SYT8* interaction in islets, we measured its effect on 3C contacts. Before glucose addition, interactions of *SYT8* and its nearby region with *INS* were readily detected; half an hour after glucose addition, *INS* interactions with the *SYT8* promoter and its 3′ downstream region (or the *TNNI2* promoter) were increased by 3–5 fold in islets from two donors (Fig. 2a). The *INS-CTSD* interaction, in contrast, was weak both before and after glucose treatment (Fig. 2a). One hour after glucose treatment, a 1.8- to 3.2-fold increase in *INS-SYT8* interaction was also seen in islets from three donors (Fig. 2b). Time course analysis in one donor's islets showed that this interaction increased five fold within 30 min of glucose treatment and then gradually decreased (Fig. 2c). We conclude that glucose dynamically regulates interactions of the *SYT8-TNNI2* locus with *INS* in islets.

Glucose markedly increases human *INS* promoter activity in primary cultured islet cells[13,14] and also stimulates endogenous *INS* gene transcription in human islets[15,16]. It has been reported that, within 30 min of addition, glucose markedly increases H4 acetylation and RNA polymerase II recruitment to the *INS* promoter in islets[11], while the levels of mature *INS* transcripts appear to be stable for at least 48 hours after glucose treatment; their long half-life leads to high levels of accumulation in β cells[15,16]. We confirmed this result: levels of mature *INS* transcript were stable (Fig. 2d). On the other hand, steady-state mRNA levels of *SYT8* and *TNNI2* were increased by 1.4- to 3.2-fold within 30 min of glucose treatment in islets from 3 donors (Fig. 2d). In contrast, *SYT7* and *SYT13* expression levels were almost unchanged (Fig. 2d). Thus, glucose specifically and rapidly increases *SYT8* and *TNNI2* gene expression in islets, with kinetics of mRNA accumulation similar to the *EGR1* (*ZIF268*) gene, whose expression is also regulated by glucose in pancreatic β cells[33] (Supplementary Fig. 5). Therefore, *SYT8* and *TNNI2* increases in expression correlate in time with increases in *INS* interactions with the *SYT8* and *TNNI2* locus upon glucose treatment in islets.

The *INS* promoter positively regulates *SYT8* and *TNNI2* expression

The data presented so far are formally consistent with a model in which both *INS* and *SYT8* expression are stimulated by glucose, and as a consequence are drawn into proximity at a shared transcription factory, as shown for the globin locus[34,35]. The data are also consistent with a distinct, but not incompatible, model in which insulin expression levels directly affect

SYT8 expression. To investigate the role of the *INS* promoter in *SYT8* and *TNNI2* transcription, we treated islets with siRNAs that specifically target the *INS* promoter (Fig. 3a). Promoter-targeting siRNAs have been shown to inactivate endogenous promoters in dividing and non-dividing human cells[36,37]. Islets were treated with the *INS* promoter-targeting siRNA duplexes or control siRNA duplexes for four and a half days. To prevent effects of the potential insulin deficiency in siRNA-treated islets on gene expression[38], siRNA-treated islets were washed twice with fresh basal islet media and cultured in fresh supplemented islet media containing physiological levels of exogenous insulin for at least 6 h before total RNAs were prepared. Treatment of islets from two donors with these siRNAs led to 34–50% reduction in the *INS* preRNA levels (Fig. 3a, b), indicative of effective silencing of the *INS* promoter. The steady-state *INS* mRNA levels were stable or slightly decreased after this short-term siRNA treatment (Fig. 3a, b) probably because of their long half-life. This siRNA-induced inactivation of the *INS* promoter decreased *SYT8* and *TNNI2* gene expression by 58–83% and 43–66%, respectively, in islets from two donors (Fig. 3a, b) but did not alter expression of *SYT13* or of *CTSD* and *MRPL23*, two genes located about 60 and 100 kb away, respectively, from each end of the *SYT8-TNNI2* locus. In addition, this inactivation of the *INS* promoter had little effect on expression of *KCNQ1* and *TSSC4*, which are also located in the 1-Mb region (Supplemental Fig. 6a). These results demonstrate that the *INS* promoter exerts long-range effects on the regulation of *SYT8* and *TNNI2* gene transcription in islets. Although it is likely that *INS*, *SYT8* and *TNNI2* do share a transcription factory, this is not sufficient to explain the dependence of *SYT8* expression on the *INS* promoter (see Discussion). The data also indicate that long-range regulation of transcription by the *INS* promoter is not domain-wide but rather gene-specific. Since inactivation of the *INS* promoter has no effect on expression of *MRPL23*, a gene located between the *SYT8* and *INS* loci and about 100 kb closer to *INS* than *SYT8* and *TNNI2*, the *INS* promoter seems not to spread its effects on transcription by linear propagation of an activating signal, but more likely through higher-order chromatin organization[1–5,20,22,24,25].

The mechanism by which the *INS* promoter transactivates *SYT8* remains to be determined. However, we found that the *INS* promoter, when coupled to the *SYT8* promoter, could stimulate *SYT8* promoter activity (Supplementary Fig. 6b). In a complementary experiment, inactivating the *SYT8* promoter in islets had little effect on *INS* expression (Supplementary Fig. 7).

CTCF regulates *SYT8* and *TNNI2* expression and their contacts with *INS*

CTCF binds to DNA, largely in a cell-type-independent manner[39] and is a major regulator of chromatin architecture[20,22,24,39]. The presence of CTCF-binding sites at both *SYT8* and *TNNI2* promoters and at the 3′ downstream region of the *INS* gene[40] (see Supplementary Fig. 8a, b for the location) suggests its involvement in the regulation of *INS-SYT8* interaction. Incubation of islets with *CTCF*-specific siRNAs reduced *CTCF* gene expression from four donors by 40–72% (Fig. 4a) while it caused a 46–76% decrease in *SYT8* mRNA levels, but had little effect on *SYT7* and *INS* gene expression (Fig. 4a). Similarly to *SYT8*, *TNNI2* gene expression was also reduced by *CTCF* knockdown in islets from three of four donors (Fig. 4a). *CTCF* depletion reduced *SYT8-INS* interaction in the same islets described above (Fig. 4b and Supplementary Fig. 8c). The decrease in *SYT8-INS* interaction and

SYT8 gene expression is correlated with the extent of *CTCF* knockdown (Fig. 4a, b), indicating that *CTCF* is important for maintenance of *SYT8-INS* interaction as well as *SYT8* and *TNNI2* gene expression in islets. The *INS-SYT8* interaction is also detected, although at much lower levels (Fig. 1d and Supplementary Fig. 4c), in human primary fibroblasts that do not express *SYT8* and *TNNI2* genes at appreciable levels (Supplementary Fig. 4b). *CTCF* depletion in fibroblasts reduced CTCF binding at the *SYT8* and *INS* loci and caused a 67% reduction in the *SYT8-INS* interaction (Fig. 4c-e), suggesting that CTCF binding at the *SYT8* and *INS* loci also regulates long-range *SYT8-INS* interactions in human fibroblasts.

SYT8 is an important regulator of insulin secretion in islets

The existence of an actively-regulated mechanism for *SYT8* gene expression (Fig. 2 and Fig. 3) and its potential involvement in insulin secretion[26] suggest a role of *SYT8* in controlling glucose response in islets. Incubation of islets with an *SYT8*-specific siRNA caused a 77% reduction in *SYT8* gene expression (Fig. 5a). Compared to islets treated with control siRNA, basal insulin secretion from *SYT8*-depleted human islets was decreased on average by 43% ($P<0.0001$) (Fig. 5b). *SYT8* knockdown in islets from two donors also attenuated glucose- and arginine-induced insulin secretion by more than 50% ($P<0.0001$) (Fig. 5c, d) and 31% ($P=0.0018$) (Fig. 5d), respectively. Finally, *SYT8* depletion impaired the first and second phase of insulin secretion in an insulin secretion assay (Fig. 5e). We conclude that, similar to *Syt7* in mice[30], the *SYT8* gene is important for both basal and glucose-stimulated insulin secretion in human islets. Consistent with this finding, the mouse *Syt8* gene is found important for exocytosis of acrosomes in sperm[41].

DISCUSSION

An important role for the *INS* promoter in long-range transcriptional regulation was evident from our observations that specific inhibition of *INS* transcription by promoter-targeting siRNA decreases *SYT8* and *TNNI2* gene expression by 40–80% in human islets. *SYT8* and *TNNI2* gene expression correlate positively with their interactions with the *INS* gene in islets treated with glucose (Fig. 2), indicating a positive role for intranuclear contact between the *INS* gene and the *SYT8-TNNI2* locus in *SYT8* and *TNNI2* gene transcription. Glucose stimulates both *INS* promoter activity in primary islet cells[13,14] and interactions of the *INS* gene with the *SYT8-TNNI2* locus in islets (Fig. 2), compatible with the notion that glucose could enhance *SYT8* and *TNNI2* gene expression through increasing their interactions with *INS*. This study reveals a mechanism by which a strong tissue- or cell-type-specific regulatory element can coordinate cellular response to acute environmental and developmental signals through remote control of transcription of a second broadly expressed gene.

Our work indicates that CTCF is important for maintenance of interactions of *INS* with *SYT8* and *TNNI2* and for *SYT8* and *TNNI2* expression in islets. Since *CTCF* depletion had little effect on *INS* gene expression in human islets (Fig. 4a), CTCF probably does not act by directly activating transcription but more likely through regulating long-range chromosomal interactions as previously shown in the β-globin and *H19* loci[20,22,24,25]. The observations

that *SYT8-INS* interactions and *SYT8* gene expression are markedly reduced upon *CTCF* depletion and that CTCF-binding sites are present in both *INS* and *SYT8* loci suggests that CTCF-dependent *SYT8-INS* interactions positively regulate *SYT8* and *TNNI2* gene transcription in islets. CTCF may be involved in many cell types in organizing local chromatin architecture that could poise the *SYT8* and *TNNI2* genes for enhanced transcriptional activation once a cell type-specific activating signal is available (See model in Supplementary Fig. 9). Due to technical limitations in use of human tissues and potential indirect effects of *CTCF* knockdown, we can only demonstrate a correlation between CTCF mediated stabilization of long range contacts and effects on *SYT8* and *TNNI2* expression. Given the complex nature of networks stabilized by CTCF interactions, it is in any case difficult to isolate the effects of a single interacting pair of sites.

Interactions of the *SYT8-TNNI2* locus with the *INS* gene may potentially promote *SYT8* and *TNNI2* gene transcription by recruiting to the *SYT8* and *TNNI2* promoters active histone marks present on the *INS* promoter[15,32,42], or by allowing transcription factors bound to the *INS* promoter to directly activate *SYT8* expression in a manner analogous to transvection in *Drosophila*. We considered whether these effects could have arisen solely from independent recruitment of both *INS* and *SYT8* promoters to a center of high RNA polymerase II concentration[34,35]. It is likely that these genes, when expressed, do share such a transcription factory. However, that is not sufficient to explain our observations that inactivation of the *INS* promoter reduces *SYT8* and *TNNI2* expression as well as *INS* gene transcription, and that *INS* expression is not affected by loss of CTCF binding and the *SYT8-INS* interaction, whereas *SYT8* and *TNNI2* expression are markedly decreased. Previously reported properties of transcription factories do not include the ability of some genes present within a factory to transactivate others in the same factory. In the case of the *INS* locus it appears that CTCF plays a role in facilitating the *INS-SYT8/TNNI2* interaction, and that this interaction is coupled with an activation of *SYT8/TNNI2* expression dependent on the level of *INS* expression. Finally, as impaired insulin secretion and diabetes have been observed in an individual with a balanced chromosomal translocation t(1;11)(p36.22;p15.5) that causes physical separation of the *INS* gene from *SYT8* and other nearby genes[43], local genomic organization around *INS* might contribute to regulation of islet function.

An important role for SYT8 in insulin secretion, identified by this study, is unexpected because SYT8 lacks the critical aspartate residues for Ca^{2+} binding at both C_2 domains and cannot bind phospholipids and target membrane SNARE (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor)[44]. In contrast to Syt7 and Syt9, Syt8 fails to stimulate *in vitro* SNARE-mediated membrane fusion[44], an essential step for exocytosis of insulin. On the other hand, SYT8 has been shown to bind syntaxins with an affinity similar to or higher than Syt1[45], to regulate *in vitro* Syt1-stimulated membrane fusion[44] and to interact with Syt7 in a Ca^{2+}-dependent manner although the interaction is weak and likely transient and hard to detect in cells[46]. These studies suggest that SYT8 may serve as a regulatory protein for other SYTs. The exact steps at which SYT8 acts during insulin secretion are unclear.

This work reveals that, through regulation of *SYT8*, the *INS* promoter may couple *INS* gene transcription with insulin secretion in islets. It seems advantageous that *SYT8* gene

transcription could be quickly adjusted in response to glucose through the *INS* promoter so that the cytosolic SYT8 pool in β cells could be refilled for the next round of insulin secretion. This novel mechanism for co-regulation is distinct from, and more direct than, the feedback regulatory effects of secreted insulin on β cells[38,47]. Together with the finding that secreted insulin stimulates *INS* gene transcription[38,47], our data reveal a positive feedback circuit that functions in islets to coordinate insulin transcription and secretion. This regulatory circuit might be important for robust and timely secretion of insulin in response to rapid changes of glucose concentrations. Furthermore, severe reduction in insulin transcription could potentially disrupt the regulatory circuit and lead to deregulation of insulin secretion. This may be relevant to pathogenesis of human diabetes: inactivating mutations in the *INS* promoter regulator genes *PDX1*, *HNF-1α*, *HNF-1β*, *HNF-4α* and *NEUROD1*[48–50] cause impaired insulin secretion in patients with monogenic forms of diabetes, maturity-onset diabetes of the young (MODY)[51], and loss-of-function mutations in the human *INS* promoter region cause neonatal diabetes[52].

Coordinated regulation of transcription by the *INS* promoter may not be limited to *SYT8*. Steady-state *TNNI2* expression is similarly regulated in islets. This suggests that glucose-regulated *INS* promoter activity may coordinate multiple β cell functions. Since TNNI2 interacts with and activates the transcriptional activity of estrogen receptor-related receptor α (ERRα)[53], an important regulator of energy metabolism[54,55], regulation of *TNNI2* gene transcription by the *INS* promoter might contribute to this function in human islets. Remote regulatory control by the *INS* promoter may have implications for pathology and treatment of type 2 diabetes. The UKPDS (United Kingdom Prospective Diabetes Study) clinical trials have shown the critical role of preserving β cell functions for the management of type 2 diabetes[56]. Our work suggests that recovering and sustaining active transcriptional activity on the *INS* promoter could constitute an important target to achieve this.

METHODS

Culture of human islets.

Isolated human pancreatic islets from independent cadaver donors were obtained from the Islet Cell Resource Centers (ICRs). Human islets were cultured in the shipping media for 2 h and placed on a 40 μm cell strainer (BD Falcon) to get rid of the dead or single islet cells. Islets on the cell strainer were washed twice with the fresh basal islet media, CMRL-1066 (99–663-cv, Cellgro) and then cultured in fresh media for at least 3 h or overnight in a 37°C cell incubator before further experiments.

Culture of human primary cells.

Human islet-derived precursor cells (hIPCs) were generated from human islets as previously described[32]. Normal human primary fibroblasts (NHPF) were obtained from ATCC and cultured as recommended.

3C analysis.

Dissociated single islet cells from 10,000 IEQs (islet equivalents) were suspended in ice-cold cross-linking buffer (10 mM Tris-HCl pH8.0, 10 mM $MgCl_2$, 50 mM NaCl) containing

0.1% NP40 and proteinase inhibitors. Fresh formaldehyde was immediately added to 2% and the islet cells were incubated on ice for 15 min. After BglII digestion overnight, 3C-ligated DNA was prepared as previously described[57]. BglII digestion of cross-linked nuclei was efficient under this condition (Supplementary Fig. 2c). The duplex PCR reactions were run with PicoMaxx High Fidelity PCR Master Mix (Stratagene) and primers for β-actin (*ACTB*), *INS* and *SYT8*. PCR products were resolved on 2% ethidium bromide-containing agarose gel. The sequences of the primers used in this work are provided in Supplementary Fig. 12.

Quantitative 3C analysis.

60 ng of 3C ligated DNA was first preamplified for 25 cycles using primers of *ACTB* or *SYT8-INS* as above. After passing through a G25 column (GE Healthcare), the preamplified DNA was diluted 100-fold. TaqMan PCR reactions were then run with TaqMan Universal PCR Master Mix (Applied Biosystems). Relative interactions were determined by using a standard curve generated by a serial dilution of the religated BglII-digested templates, which were made by mixing equal molar amount of the corresponding purified PCR products[57]. The interactions in different 3C samples were normalized to that of the *ACTB* internal control. In control experiments, serial dilutions of 3C ligated DNA were preamplified and then quantitated by TaqMan qPCR. As shown in Supplementary Fig. 10, the *SYT8-INS* interaction or the *ACTB* control interaction in the preamplified DNA precisely reflects those in the non-amplified templates.

4C-Seq analysis.

4C analysis was done as previously described[24] except for the last few steps (see details in Supplementary Fig. 2). In brief, after reverse cross-linking, 3C ligated DNAs were digested with NlaIII and then re-ligated to make circular DNA. The first inverse PCR reaction was run for 23 cycles with Phusion DNA polymerase (Finnzymes) and one 5′ biotinylated primer. The PCR products were then digested with AvrII to cut the self-ligated products. The AvrII-digested DNA was incubated with streptavidin dynabeads and DNA-bound dynabeads were subject to nested PCR using two 5′ biotinylated primers. The final PCR products were digested with BglII and NlaIII. After incubation with streptavidin dynabeads, the known bait sequence located at both ends bound to dynabeads and was discarded while the unknown bait-interacting DNA sequences present in the supernatants were purified for preparation of the sequencing library. Solexa DNA sequencing analysis was performed as previously described[40]. The sequence tag profile obtained from 4C-Seq was uploaded into UCSC Genome Browser, and DNA sequences and the chromosome location of the 4C peaks were then downloaded from the UCSC Genome Browser. As shown in Supplementary Fig. 11a, 4C peaks surrounding the *SYT8-TNNI2* locus with frequency larger than 150 all have BglII sites and NlaIII sites. 4C PCR products generated at the step prior to BglII and NlaIII digestion were also cloned and sequenced. Ten out of ten such clones had *INS* bait DNA sequence at both ends and interacting DNA sequence in the middle with BglII and NlaIII restriction sites connecting these two sequences (Supplementary Fig. 11b).

Inactivation of the *INS* promoter in human islets by promoter-targeting siRNA

Human islets were incubated at 1000 IEQs per ml of the basal islet media in non tissue-culture treated plates for four and a half days with 1 μM of one of two promoter-targeting Accell siRNA duplexes[58] (GGGAAAUGGUCCGGAAAUUUU and GGAUAGGGGUGUGGGGACAUU, Dharmacon) or the non-targeting control Accell siRNA pool (D-001910–10, Dharmacon). siRNA-treated islets were washed twice with fresh basal media and cultured in the fresh supplemented media containing insulin (99–603-CV, Cellgro) for at least 6 h before total cellular RNA was prepared.

Gene knockdown in intact human islets and normal human primary fibroblasts.

Human islets were incubated with 1 μM of Accell siRNA duplex(es) (a mixture of equal molar concentrations of GCCUUAUGAAUGUUAUAUU and GCUCUAAGAAAGAAGAUUC for *CTCF*; GGCUUAUUCCAGACCUUGU for *SYT8*) for four and a half days. siRNA-treated islets were washed twice with fresh media and cultured in the fresh media for 5 h before total cellular RNA was prepared. Normal human primary fibroblasts were incubated with 1 μM of *CTCF*-specific or control siRNA duplexes as above in Accell siRNA delivery media (Dharmacon) for 4 days.

Quantitative real-time RT-PCR analysis.

Total cellular RNA was prepared using RNeasy Mini reagents (Qiagen), and residual genomic DNA was removed by DNase I treatment using a DNA-free kit (Ambion). DNase-treated RNA was then converted to cDNA using the AffinityScript QPCR cDNA synthesis kit (Stratagene) and analyzed by real-time PCR using POWER SYBR Green PCR Master Mix (Applied Biosystems). Relative levels of cDNAs were determined by using a standard curve generated by serial dilutions of cDNAs. *INS E2I2* preRNA levels and steady-state levels of *SYT8*, *SYT7*, *SYT13*, *INS*, *CTCF*, *TNNI2*, *CTSD*, and *MRPL23* mRNAs were normalized to those of *HPRT1*. Each qRT-PCR assay was done in triplicate and independently repeated three times.

Islet insulin secretion experiments.

After siRNA treatment, islets were washed twice and suspended in fresh media and cultured for 3 h. 25 mM D-glucose or 20 mM L-arginine was added to the media for 30 min. Islets on 40 μm cell strainers were also placed into wells containing 5 ml of fresh stimulating media (containing 30.5 mM glucose) and were moved into a new well every 5 min. Finally, human islets were spun down and supernatants collected while total cellular RNA was prepared from the pellets of human islets. The insulin levels in the islet media were determined using a human insulin ELISA assay (IS130D, Calbiotech) and were normalized to the amount of total RNA prepared from the islets. Each ELISA assay was done in duplicate and repeated twice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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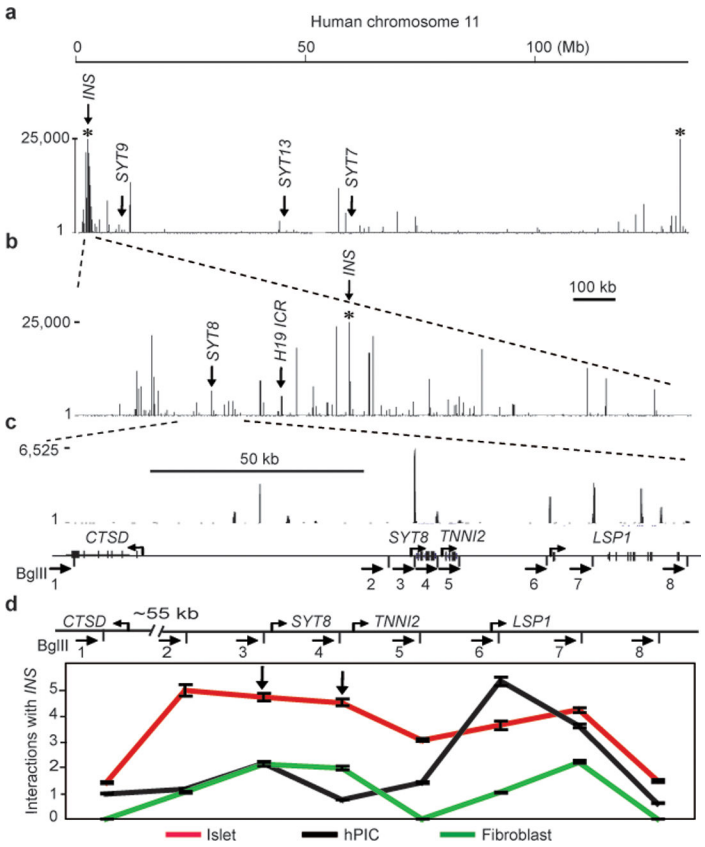

Figure 1.

4C-Seq analysis reveals the association of *SYT8* with *INS* gene in human pancreatic islets. 4C-Seq analysis of *INS*-associated loci in the entire human chromosome 11 (**a**), the *INS* nearby region (**b**) and the *SYT8–TNNI2* gene locus (**c**). 4C peaks are shown (**a-c**), and *INS*, *SYT9*, *SYT8*, *SYT13* and *SYT7* genes and the *H19 ICR* are located (**a**, **b**). The frequency of two 4C peaks marked with asterisks is larger than 25,000 and truncated. (**c**) The location of *SYT8* and *TNNI2* as well as *CTSD* and *LSP1* genes, the 3C PCR primers and the corresponding BglII sites (vertical line) in the *SYT8* and *TNNI2* locus (*lower*). The 3C PCR primers are numbered. The exons are marked with solid bars and the transcription direction marked with arrows. Not all of the BglII sites in this region are shown and there is only one BglII site within the *CTSD* gene. (**d**) TaqMan quantitative 3C analysis of *INS* interactions with the *SYT8-TNNI2* locus in glucose-treated islets, islets-derived hIPCs and primary human fibroblasts. Plotted are the relative interactions in arbitrary units of the *INS* gene with eight BglII sites (*upper*, not in scale), which are numbered as in **c** and whose location in the region shown in **c**. The mean ± SEM is shown ($n = 8$). The sites for the *SYT8* promoter and its 3′ downstream site (or the *TNNI2* promoter) are marked with arrows.

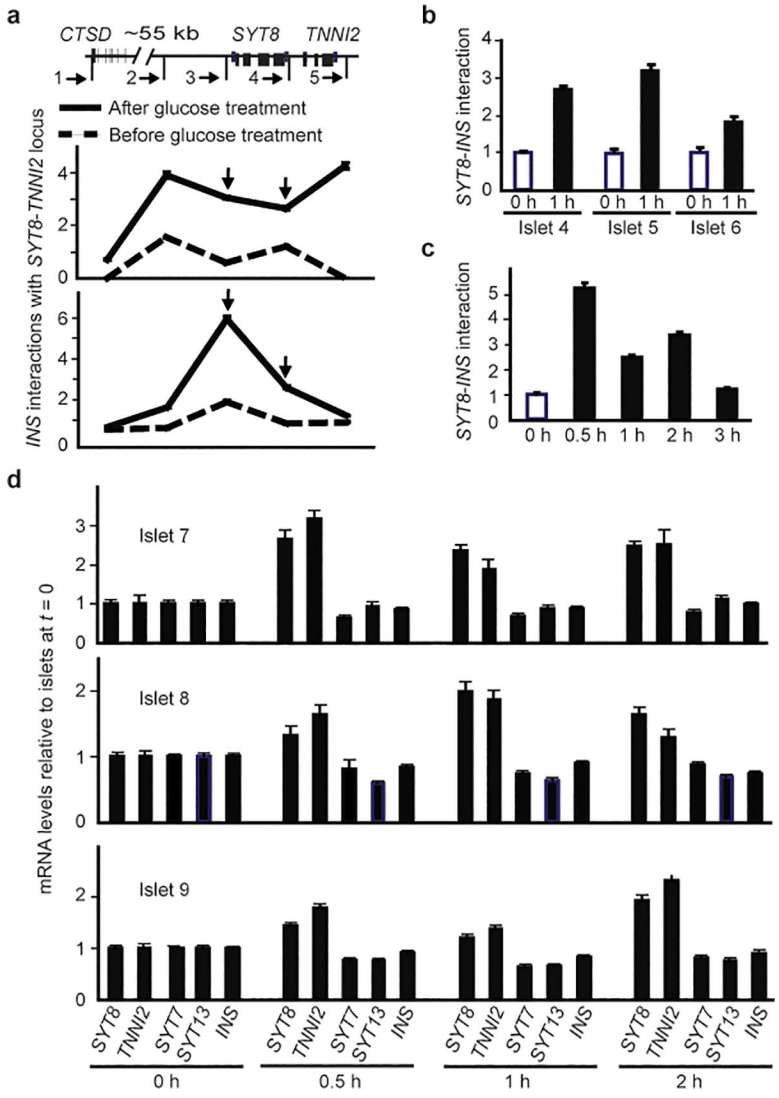

Figure 2.

Glucose stimulates *INS* interactions with the *SYT8-TNNI2* gene locus and increases *SYT8* and *TNNI2* gene expression in human islets. (**a**) TaqMan quantitative 3C analysis of the relative interaction of the *INS* gene with the *SYT8* promoter and its 3′ downstream site (or the *TNNI2* promoter), marked with arrows, in islets from two donors with and without glucose treatment for 30 min. The mean ± SEM is shown ($n = 8$). (**b**) TaqMan quantitative 3C analysis of the interaction of the *INS* gene with the *SYT8* promoter in islets from three donors with and without glucose treatment for 1 h. The *SYT8-INS* interaction is shown relative to that in islets without glucose treatment. The mean ± SEM is shown ($n = 8$). (**c**) Time-course analysis of the interaction of the *INS* gene with the *SYT8* promoter in islets from one other donor treated with glucose for the indicated times. (**d**) Quantitative real-time RT-PCR (qRT-PCR) analysis of *SYT8*, *TNNI2*, *SYT7*, *SYT13* and *INS* gene expression in islets from three donors with and without glucose treatment for the indicated times. The RNA levels are normalized to those of *HPRT1* and the mRNA levels relative to islets at $t = 0$ are plotted. The mean ± SEM is shown ($n = 9$).

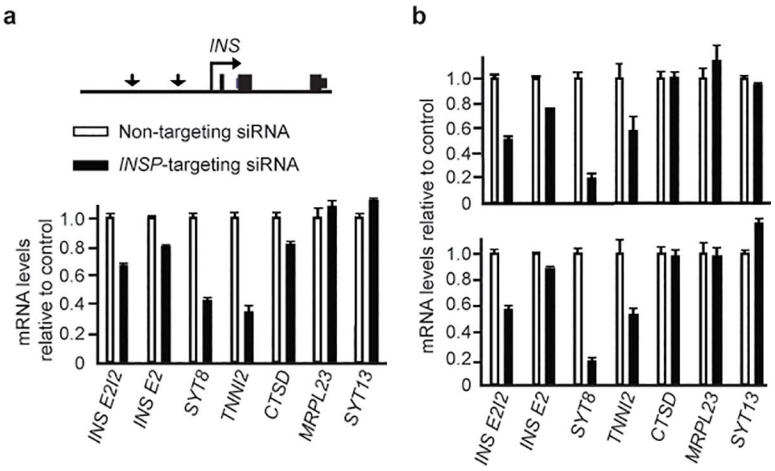

Figure 3.

The *INS* promoter positively regulates *SYT8* and *TNNI2* gene expression in human islets. (**a**) qRT-PCR analysis of *INS* preRNA *E2I2* and mature transcript *INS E2* (exon 2) and mature transcripts of *SYT8*, *TNNI2*, *CTSD*, *MRPL23* and *SYT13* genes in islets from two donors that were treated with non-targeting control siRNA (white bar) or one of the two siRNAs targeting to the *INS* promoter (black bar). The RNA levels are normalized to those of *HPRT1*. mRNA levels are plotted relative to control. The mean ± SEM is shown ($n = 9$). The *INS* exons are shown and the siRNA-targeting sites are marked with arrows (*top*). (**b**) qRT-PCR analysis as in **a** of the islets from one other donor that were treated separately with either of the two siRNAs targeting to the *INS* promoter.

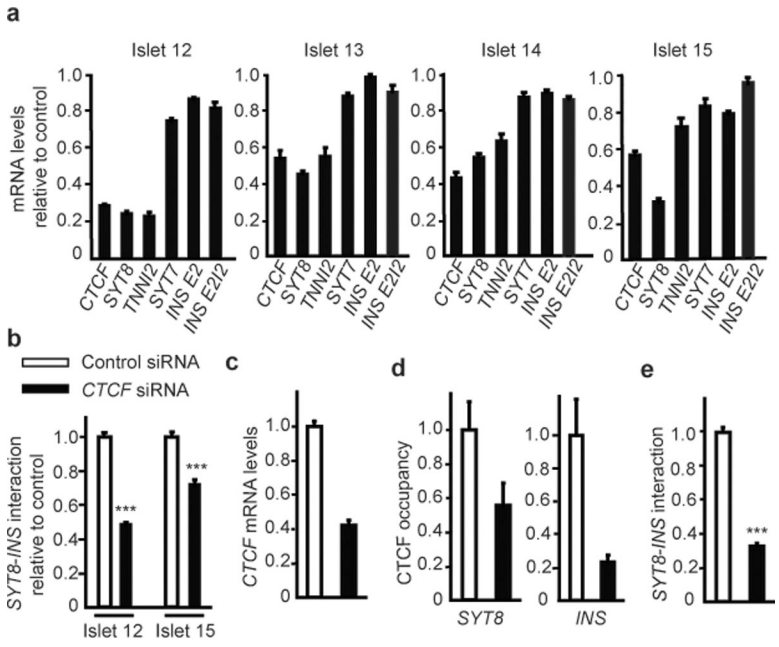

Figure 4.

CTCF positively regulates *SYT8* and *TNNI2* gene expression in human islets and is important for the maintenance of the *SYT8-INS* interaction in human islets and human fibroblasts. (**a**) qRT-PCR analysis of *CTCF*, *SYT8*, *TNNI2*, *SYT7* and *INS* gene expression in islets from four donors that were treated with non-targeting control or *CTCF*-specific siRNA. The RNA levels are normalized to those of *HPRT1*. Plotted are the mRNA levels relative to control. The mean ± SEM is shown ($n = 9$). (**b**) TaqMan quantitative 3C analysis of *INS-SYT8* interaction in the two same siRNA-treated islets as shown in **a**. The *SYT8-INS* interaction is shown relative to control. ***P < 0.0001. (**c**) qRT-PCR analysis of *CTCF* gene expression in normal human primary fibroblasts that were treated with control (white bar) or *CTCF*-specific siRNA (black bar). Shown are the *CTCF* mRNA levels relative to control. (**d**) Quantitative ChIP analysis of CTCF occupancy at the *SYT8* promoter and the *INS* 3′ downstream region in human fibroblasts treated as in **c**. Shown is the CTCF occupancy relative to control. (**e**) TaqMan quantitative 3C analysis of *INS-SYT8* interaction in human fibroblasts treated as in **c**. Shown is the *SYT8-INS* interaction relative to control. ***P < 0.0001.

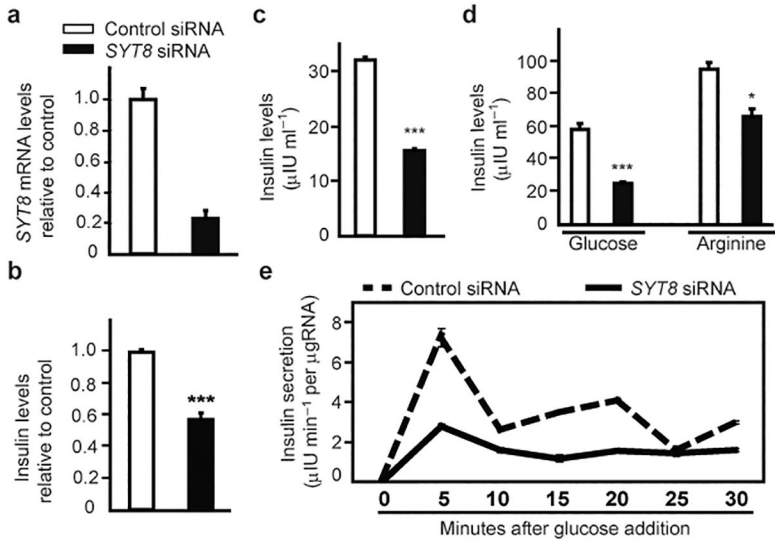

Figure 5.

SYT8 is an important regulator of insulin secretion in human islets. (**a**) qRT-PCR analysis of *SYT8* gene expression in islets treated with control (white bar) or *SYT8*-specific siRNA (black bar). Total RNA was prepared directly from siRNA-treated islets without medium change. The mean ± SEM is shown ($n = 9$). (**b**) ELISA analysis of insulin levels in the medium for islets from 5 donors that were separately treated with siRNA as in **a**. The insulin levels are normalized to the amount of total RNAs made from the treated islets. As insulin levels vary among islets from different donors, the insulin levels relative to control are plotted. The mean ± SEM is shown ($n = 20$, 4 measures for each of 5 donors); ***P < 0.0001. (**c**) ELISA analysis of insulin levels in the medium of islets from one donor treated as in **b** and cultured with 30.5 mM glucose for 30 min. The insulin levels are normalized to the amount of islets necessary to produce 1μg total RNA; ***P < 0.0001. (**d**) ELISA analysis of insulin levels in the medium of islets from another donor that were treated as in **c** and cultured with 30.5 mM glucose or 20 mM L-arginine for 30 min. The insulin levels are normalized as in **c**.***P<0.0001; *P=0.0018. (**e**) Insulin secretion from siRNA-treated islets that were treated with 30.5 mM glucose and supernatants were collected every 5 min. The mean ± SD is shown ($n = 4$).