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TCF7L2 is a master regulator of insulin production and processing

Yuedan Zhou[1], Soo-Young Park[2], Jing Su[4], Kathleen Bailey[3], Emilia Ottosson-Laakso[1], Liliya Shcherbina[1], Nikolay Oskolkov[1], Enming Zhang[1], Thomas Thevenin[1], João Fadista[1], Hedvig Bennet[1], Petter Vikman[1], Nils Wierup[1], Malin Fex[1], Johan Rung[5], Claes Wollheim[1,6], Marcelo Nobrega[3], Erik Renström[1], Leif Groop[1] and Ola Hansson[1,*]

[1]Department of Clinical Sciences, CRC, Lund University, Malmö 20502, Sweden, [2]Department of Medicine and [3]Department of Human Genetics, University of Chicago, IL 60637, USA, [4]European Bioinformatics Institute, Functional Genomics, Hinxton, Cambridge CB10 1SD, UK, [5]Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala 75185, Sweden and [6]Department of Cell Physiology and Metabolism, Université de Genève, University Medical Centre, 1 rue Michel-Servet, Geneva 4 1211, Switzerland

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Genome-wide association studies have revealed >60 loci associated with type 2 diabetes (T2D), but the underlying causal variants and functional mechanisms remain largely elusive. Although variants in *TCF7L2* confer the strongest risk of T2D among common variants by presumed effects on islet function, the molecular mechanisms are not yet well understood. Using RNA-sequencing, we have identified a TCF7L2-regulated transcriptional network responsible for its effect on insulin secretion in rodent and human pancreatic islets. *ISL1* is a primary target of TCF7L2 and regulates proinsulin production and processing via *MAFA*, *PDX1*, *NKX6.1*, *PCSK1*, *PCSK2* and *SLC30A8*, thereby providing evidence for a coordinated regulation of insulin production and processing. The risk T-allele of rs7903146 was associated with increased *TCF7L2* expression, and decreased insulin content and secretion. Using gene expression profiles of 66 human pancreatic islets donors', we also show that the identified TCF7L2-ISL1 transcriptional network is regulated in a genotype-dependent manner. Taken together, these results demonstrate that not only synthesis of proinsulin is regulated by TCF7L2 but also processing and possibly clearance of proinsulin and insulin. These multiple targets in key pathways may explain why *TCF7L2* has emerged as the gene showing one of the strongest associations with T2D.

INTRODUCTION

TCF7L2 harbors common genetic variants with the strongest effect on type 2 diabetes (T2D) risk yet described, i.e. the single-nucleotide polymorphism (SNP rs7903146) (1–3). TCF7L2 is a transcription factor (TF) in the Wnt-signaling pathway and expressed in many tissues including fat, liver and pancreatic islets of Langerhans (4). A majority of risk variants associated with T2D by GWAS seem to affect islet function (5,6). In concordance, the risk T-allele of rs7903146 is associated with impaired glucose-stimulated insulin secretion (GSIS) or other secretagogues-like GLP-1 (2,7–12). Risk T-allele carriers are further characterized by an elevated plasma proinsulin level and an increased proinsulin-to-insulin ratio suggestive of perturbed proinsulin processing (13–21). In support of a primary effect in pancreatic islets, the risk T-allele carriers show higher degree of open chromatin in pancreatic islets, but not in other tissues (22). Several mouse models of *Tcf7l2* have been investigated (23–28), but the diabetic phenotype has not been replicated in all studies. Experiments in rodent islets where TCF7L2 activity has been disrupted have usually demonstrated impaired GSIS (11,29,30).

*To whom correspondence should be addressed at: Department of Clinical Science, Endocrinology and Diabetes, Lund University, Ing 72, Building 91, Fl 12, Sweden. Tel: +46-391228; Fax: +46-391222; Email: Ola.Hansson@med.lu.se

Paradoxically, carriers of the risk T-allele show increased expression of *TCF7L2* in human pancreatic islets (7,31). Plasmid construct carrying the risk T-allele, when compared with those with the C-allele, displayed a much stronger transcriptional activity, implying a higher enhancer activity of the T-allele (22,32). TCF7L2 may act both as a stimulator and a repressor of gene expression due to both differential splicing and interaction with different co-regulators in target gene recognition (33). However, the target genes of TCF7L2 in pancreatic islets or B-cells have not been described in detail. Our aim was to identify TCF7L2-target genes in pancreatic islets and to describe the regulatory network downstream of *TCF7L2* responsible for its influence on insulin secretion.

RESULTS

Tcf7l2 influences insulin synthesis and secretion

Impaired *in vivo* (7) and *in vitro* (9,31) insulin secretion has been shown in risk T-allele carriers of rs7903146. We have replicated and extended these findings in pancreatic islets from human non-diabetic cadaver donors ($n = 75$, Fig. 1A; Supplementary Material, Table S3). We also show reduced insulin content (27%) in risk T-allele carriers ($n = 81$, Fig. 1B), and that *TCF7L2* mRNA expression is 16% higher than C-allele carriers ($n = 66$; RNA-seq, Fig. 1C). Further, *TCF7L2* expression is negatively associated with proinsulin expression in human islets in C-allele carriers ($n = 36$), but not in T-allele carriers ($n = 30$, Fig. 1D; Supplementary Material, Table S3).

Silencing *Tcf7l2* in a rat insulinoma cell line (INS1 832/13) using siRNA targeting the invariable exon 1, resulted in 84% knockdown of *Tcf7l2* mRNA, 90% reduction of TCF7L2 activity and a reduced expression of the well-known TCF7L2-target genes *Axin2* and *Ccnd1* (Supplementary Material, Fig. S1A and B) (30). The decrease in *Tcf7l2* resulted in a marked reduction of GSIS, i.e. 1.76-fold (*Tcf7l2* silencing, $P > 0.05$) versus 6.97-fold (controls, $P < 0.05$, Fig. 1E) and a perturbed glucose-stimulated proinsulin secretion (GSPS) ($P < 0.05$, Fig. 1F). Proinsulin-to-insulin ratio was higher under glucose stimulation compared with basal condition in *Tcf7l2* silenced cells ($P < 0.05$, Fig. 1G) suggesting perturbed proinsulin processing. Immunohistochemistry (IHC) analysis of *Tcf7l2* silenced INS1 cells showed reduced staining intensity for insulin ($P < 0.001$) and VAMP2 ($P < 0.001$), a marker of insulin-containing granules, in *Tcf7l2* silenced cells (Fig. 1H). Furthermore, there was a reduction in insulin-containing granules close to the plasma membrane as assessed by total internal reflection fluorescence (TIRF) microscopy ($P < 0.05$, Fig. 1I). Together, these data suggest that TCF7L2 mediates key aspects of insulin production, processing and intracellular trafficking.

Identification of TCF7L2-target genes influencing insulin synthesis and secretion

The molecular mechanisms underlying these findings are not clear, but an altered expression of TCF7L2-target genes is likely part of the explanation. To identify genes regulated by TCF7L2 in B-cells, we screened for global differential expression using RNA-seq in *Tcf7l2* silenced INS1 cells versus scramble-treated control. Overall, 10779 genes were actively expressed (>30 aligned paired-end reads per gene, corresponding to $> \sim 10$ FPKM); 1680 genes were upregulated and 1885 genes were downregulated in *Tcf7l2* silenced cells (5% false discovery rate, FDR, $n = 4$). Among the most differentially expressed genes were *Il6r*, *Aldh1a1* and *Srp14*, but none of these has a known function in insulin secretion. Genetic variation in the *IL6R*-locus has been weakly associated with T2D (34), but this was not replicated (35) or supported by recent GWAS findings (21,36–42); therefore, *Il6r* was not studied further. Instead we categorized genes known to influence GSIS into three groups: (i) glucose sensing, (ii) proinsulin expression and maturation and (iii) exocytotic machinery (genes differentially regulated in the RNAseq screen are reported in Table 1). In addition, genes located proximal to SNPs with replicated associations to T2D and/or plasma proinsulin were also selected for further analysis (Supplementary Material, Table S2). Real-time quantitative PCR (QPCR) confirmed the RNA-seq results for many of the tested genes (Table 1), i.e. 18 out of 29 analyzed genes were replicated in independent samples at the mRNA level. To confirm these findings at the protein level, a selected set of 15 genes (with mRNA change >30%) was analyzed using western blot, with 11 genes having significant changes (Supplementary Material, Table S3, the complete list of 3565 potential target gene can be found in the Supplementary Material, Table S6).

Tcf7l2 is a key regulator of proinsulin production and processing

Twelve analyzed genes involved in proinsulin synthesis were downregulated in *Tcf7l2* silenced cells, including proinsulin genes *Ins1* and *Ins2* (Table 1 and Fig. 2A). The reduction of all but one (*Cpe*) was confirmed using QPCR. The protein encoded by the *Ins1* and *Ins2* genes was decreased in *Tcf7l2* silenced cells (Fig. 2A). We next replicated this finding in primary cells by measuring proinsulin protein expression in a *Tcf7l2* homozygous null mouse model (*Tcf7l2*$^{-/-}$) (26). Notably, proinsulin expression was reduced by 85% ($n = 6$) in pancreas from *Tcf7l2*$^{-/-}$ mice (postnatal day 0; P0) compared with wild-type mice (Fig. 2B). Given differences in the insulin gene between rodents and humans (43), we also replicated the findings in human pancreatic islets showing 49% reduced proinsulin mRNA expression after *TCF7L2* silencing ($n = 5$, Fig. 2C).

In the processing of proinsulin to insulin, the endoprotease prohormone convertase 1/3 (PCSK1) and prohormone convertase 2 (PCSK2) cleave the proinsulin molecule to yield mature insulin and C-peptide. We found reduced mRNA expression levels of both *Pcsk1* and *Pcsk2* in the RNA-seq screen of *Tcf7l2* silenced INS1 cells (Table 1). This result was confirmed using QPCR and at the protein level in INS1 cells (Fig. 2A). No significant change in PCSK1 and PCSK2 protein expression was observed in the *Tcf7l2*$^{-/-}$ mouse pancreas (Fig. 2B). In *TCF7L2* silenced human pancreatic islets, *PCSK1* and *PCSK2* mRNA expression was reduced by 36 and 49%, respectively (Fig. 2C).

Tcf7l2 is not a major regulator of genes involved in the exocytotic machinery or glucose sensing

Of the 11 genes categorized as involved in the exocytotic machinery, mRNA expression of *Cacna1a*, *Cana1c*, *Cacna1d* and *Cacnb3* was reduced in *Tcf7l2* silenced cells (Table 1;

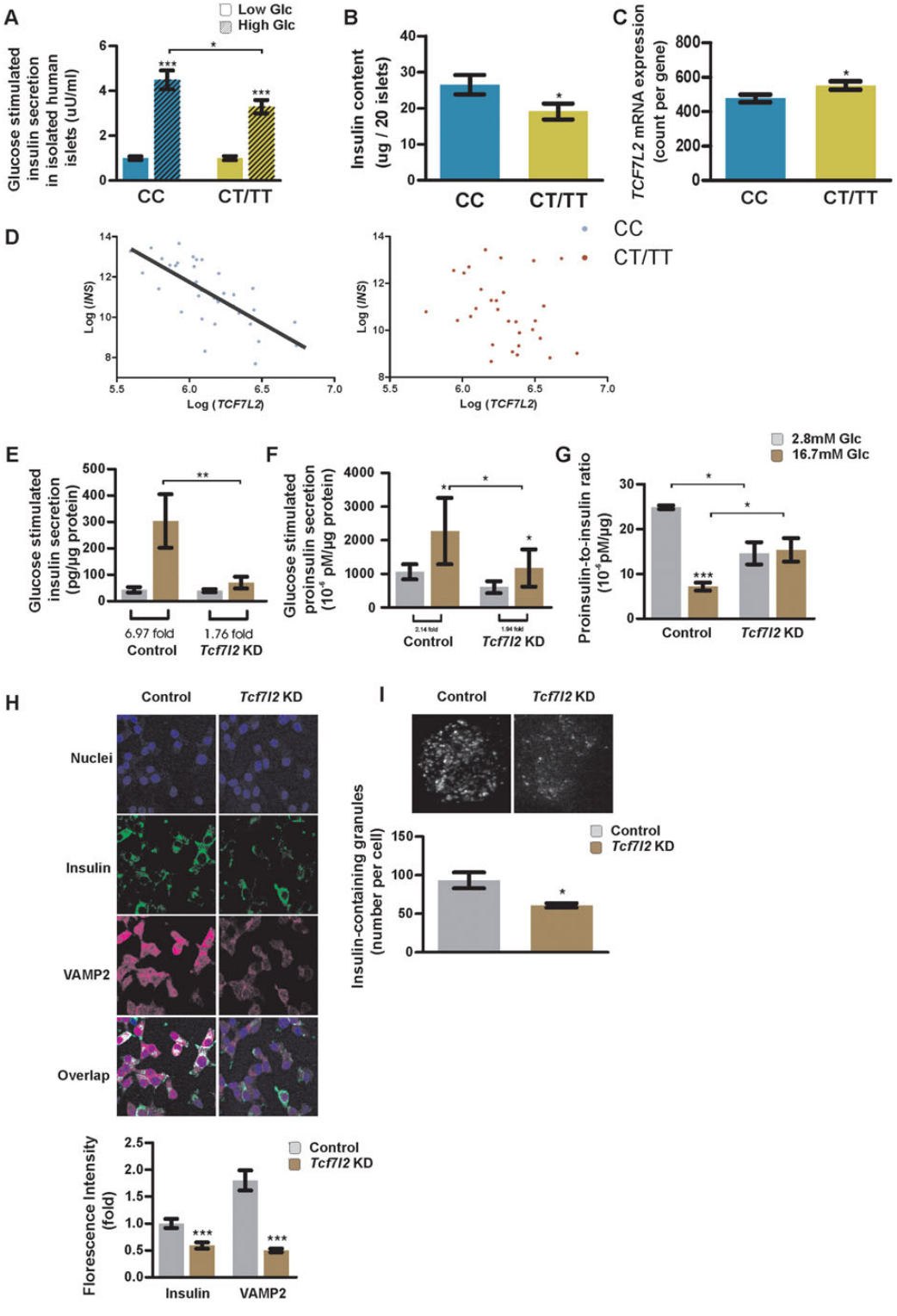

Figure 1. The type 2 diabetes-associated SNP rs7903146 and *TCF7L2* expression influences insulin synthesis and secretion. (**A**) GSIS in human islets (CC: $n = 44$, CT/TT: $n = 31$). (**B**) Insulin content in human islets (CC: $n = 45$, CT/TT: $n = 36$). (**C**) *TCF7L2* mRNA expression measured by RNA-seq in human islets (CC: $n = 36$, CT/TT: $n = 30$). (**D**) Expression (mRNA) of *INS* and *TCF7L2* in human islets measured by RNA-seq stratified for rs7903146 genotype (CC: $n = 36$, CT/TT: $n = 30$). (**E**) GSIS in INS1 cells after *Tcf7l2* silencing. (**F**) GSPS in INS1 cells after *Tcf7l2* silencing. (**G**) Proinsulin-to-insulin ratio in INS1 cells after *Tcf7l2* silencing. (**H**) IHC staining of insulin and VAMP2 in INS1 cells after *Tcf7l2* silencing. (**I**) Amount of insulin-containing granules measured by TIRF microscopy in INS1 cells after *Tcf7l2* silencing. Data are represented as mean ± SEM. In (A)–(D), data were analyzed by linear regression with correction for age, gender and BMI. In (E)–(I), 2.8 versus 16.7 mM glucose were analyzed by paired Student's *t*-tests, *Tcf7l2* silencing versus controls by unpaired Student's *t*-tests with $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Supplementary Material, Tables S1 and S4).

Table 1. mRNA expression of selected genes influencing GSIS measured using RNA sequencing (RNAseq) in *Tcf7l2* depleted INS1 832/13 cells versus scrambled control, with replication in independent experiments using real-time QPCR

	Gene symbol	Gene ID	RNAseq LogFC	*P*-value corrected	QPCR LogFC	*P*-value
Glucose sensing	*Glud1*	ENSRNOG00000010222	−0.7143	7.15E−07	−0.8681	4.97E−03
	Slc2a2	ENSRNOG00000011875	−0.5864	9.20E−06	−0.4150	2.68E−03
	Gck	ENSRNOG00000015024	−1.1232	6.26E−04	−0.0145	0.9905
	G6pc	ENSRNOG00000036806	0.3828	7.80E−04	N/A	N/A
	Mpc1	ENSRNOG00000012415	−0.4338	2.00E−03	N/A	N/A
Exocytotic machinery	*Cacna1a*	ENSRNOG00000002559	−1.0612	2.71E−12	−1.5564	6.52E−04
	Syt5	ENSRNOG00000018217	−0.9024	2.46E−10	−0.4739	4.24E−02
	Stxbp1	ENSRNOG00000015420	−0.6269	5.93E−06	−0.3401	4.51E−02
	Cacna1d	ENSRNOG00000013147	−0.6143	1.89E−05	−1.0893	1.88E−02
	Cacna1c	ENSRNOG00000007090	−0.7861	2.10E−05	−0.8625	2.20E−03
	Cacnb3	ENSRNOG00000012489	−0.5654	6.67E−04	−1.0000	4.00E−04
	Stx1a	ENSRNOG00000029165	0.5308	5.10E−04	−0.0145	0.8288
	Snap25	ENSRNOG00000006037	−0.4135	3.49E−03	−0.2863	0.2271
	Syt9	ENSRNOG00000019613	−0.4048	1.28E−02	−0.3219	0.0597
	Cacnb1	ENSRNOG00000004518	0.6164	2.76E−02	−0.0589	0.6316
	Vamp2	ENSRNOG00000006989	−0.2960	3.29E−02	−0.2176	0.2964
Proinsulin production and maturation	*Slc30a8*	ENSRNOG00000004747	−1.4601	1.08E−15	−0.8365	8.80E−03
	NeuroD1	ENSRNOG00000005609	−1.1931	9.73E−14	−1.2176	3.17E−03
	Cpe	ENSRNOG00000043387	−1.4682	3.16E−09	−0.3040	0.1358
	Isl1	ENSRNOG00000012556	−1.1075	1.23E−07	−1.3585	7.40E−04
	Ins1	ENSRNOG00000012052	−1.1761	5.38E−07	−1.3959	1.40E−02
	Pcsk2	ENSRNOG00000005438	−0.8951	9.81E−06	−0.6897	4.56E−02
	Nkx6.1	ENSRNOG00000002149	−0.6335	7.70E−05	−0.5995	9.26E−04
	Pcsk1	ENSRNOG00000011107	−0.7932	8.45E−05	−0.7859	3.45E−03
	Nkx2.2	ENSRNOG00000012728	−1.0863	4.18E−03	−0.8021	2.30E−02
	Ins2	ENSRNOG00000020405	−0.9005	5.11E−03	−1.0893	1.35E−02
	MafA	ENSRNOG00000007668	−1.1927	4.61E−02	−2.1844	2.96E−03
	Pax4	ENSRNOG00000008020	2.9857[a]	4.75E−02	N/A	N/A
	Pdx1	ENSRNOG00000046458	N/A[b]	N/A[b]	−0.8110	2.19E−02

N/A, not analyzed using QPCR; LogFC, the logarithm of fold change, i.e. *Tcf7l2* depleted/scrambled control; the *P*-value for RNAseq was corrected by FDR 5%. *P*-value for QPCR was calculated using Student's *t*-tests; $N = 4$ (RNAseq), $N = 3$ (QPCR).
[a]Very low expression.
[b]*Pdx1* was not aligned in the RNA-seq analysis.

Supplementary Material, Fig. S1C). However, this was not confirmed at the protein level for Cav2.1, Cav1.2 or Cav1.3, either 72 h or 7 days after *Tcf7l2* silencing (Supplementary Material, Fig. S1D and E). RNA expression of two out of the five genes involved in glucose sensing (*Glud1* and *Slc2a2/Glut2*) was reduced at the mRNA level in *Tcf7l2* silenced cells (Table 1), and GLUD1 protein expression was measured but no change was observed (Supplementary Material, Fig. S1F).

Tcf7l2 is regulating proinsulin expression directly via Isl1, Ins1 and indirectly via *MafA*, *NeuroD1* and *Pdx1*

After establishing that TCF7L2 is a regulator of proinsulin expression and maturation, we investigated by which molecular mechanism this may occur. For this purpose, we interrogated the RNA-seq results for genes differentially expressed between *Tcf7l2* silenced INS1 and control cells. Intriguingly, ISL LIM homeobox 1 (*Isl1*), v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (*MafA*), pancreatic and duodenal homeobox 1 (*Pdx1*), neuronal differentiation 1 (*NeuroD1*), NK6 homeobox 1 (*Nkx6.1*) and NK2 homeobox 2 (*Nkx2.2*) were all expressed at lower levels in *Tcf7l2* silenced cells (Table 1). All observations, except the NKX2.2 protein, were confirmed using QPCR and western blot (Fig. 3A). The reduction of ISL1 and NEUROD1 was further confirmed using IHC (Supplementary Material, Fig. S1G and H). These results were confirmed in *Tcf7l2*$^{-/-}$ mice, with protein levels reduced relative to wild-type mice for ISL1, MAFA and NEUROD1 (Fig. 3B). Likewise, after *TCF7L2* silencing in human islets, expression of most transcripts was downregulated, including *ISL1*, *MAFA*, *PDX1*, *NEUROD1* and *NKX6.1* (Fig. 3C). In addition, in islets from CC-genotype carriers of rs7903146, *TCF7L2* mRNA expression was negatively associated with that of *ISL1*, *MAFA* and *NKX6.1* but not for *MAFA* and *NKX6.1* in CT/TT-risk-genotype carriers (Supplementary Material, Table S4). The disruption of *Tcf7l2* resulted in a marked reduction in GSIS compared with controls 1.93- ($P > 0.05$) versus 4.10-fold ($P < 0.05$) increases (Fig. 3D). The same was observed after disruption of *Isl1*, *MafA* and *NeuroD1* (Fig. 3D).

To delineate the proinsulin regulatory cascade among the identified TCF7L2-target genes, *Isl1*, *MafA*, *Pdx1*, *NeuroD1* and *Nkx6.1* were sequentially silenced in INS1 cells using siRNA. First, knocking down *Isl1* reduced both the mRNA and protein expression of *MafA*, *Pdx1*, *Nkx6.1*, *Pcsk1*, *Pcsk2*, *Ins1* and *Ins2* (Fig. 4A). No effect of *Isl1* knockdown was observed

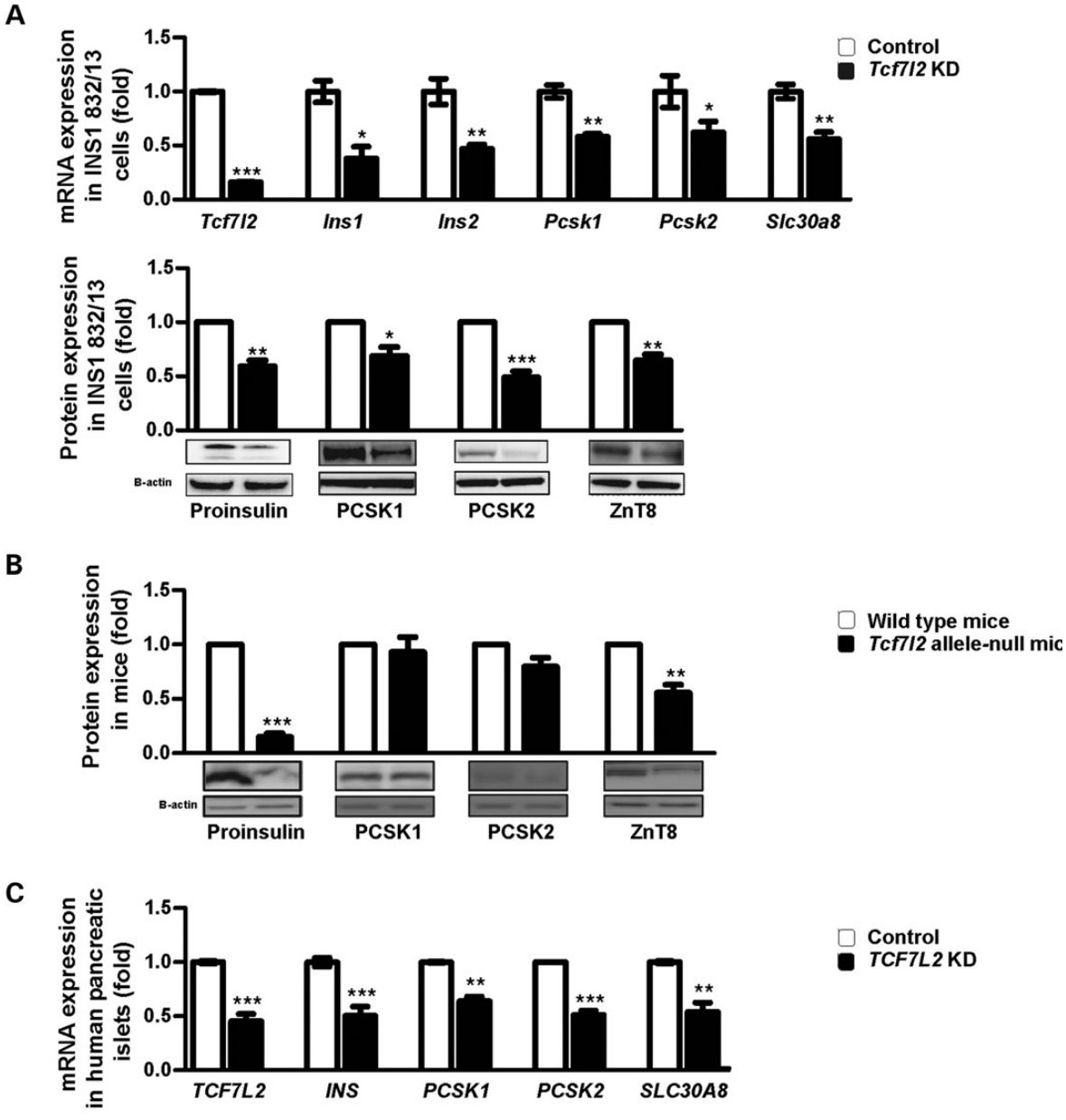

Figure 2. TCF7L2 is a key regulator of proinsulin production and processing. (**A**) mRNA expression measured by QPCR in INS1 cells in *Tcf7l2* silenced versus control cells ($n = 3$). Protein expression measured by western blot in INS1 cells in *Tcf7l2* silenced versus control cells ($n = 3$). (**B**) Protein expression measured by western blot in *Tcf7l2* allele-null versus wild-type mice ($n = 6$). (**C**) mRNA expression measured by QPCR in human pancreatic islets in *TCF7L2* silenced versus control islets ($n = 3–5$). Data are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ analyzed by Student's *t*-tests (Supplementary Material, Table S1).

on *Tcf7l2* or *NeuroD1* mRNA expression (Fig. 4A). Previously, we have analyzed chromatin immunoprecipitation on tiling array (ChIP-on-Chip) using a TCF7L2 antibody identifying both the *Isl1* and *Ins1* promoters as directly bound by TCF7L2 (30). Together, these data indicate that *Isl1* is a direct primary target gene for TCF7L2 and regulates proinsulin expression and processing via *Isl1*-dependent regulation of *MafA*, *Pdx1*, *Nkx6.1*, *Pcsk1* and *Pcsk2*. In human islets, *ISL1* expression is positively associated in C-allele carriers with that of *MAFA*, *PDX1*, *NKX6.1*, *INS* and *SLC30A8* but in T-allele carriers only with *PDX1* and *NKX6.1* (Supplementary Material, Table S4).

Knocking down *MafA* reduced mRNA expression of *Nkx6.1*, *Nkx2.2*, *Pcsk2*, *Ins1* and *Ins2*. These changes were confirmed at the protein level for all but NKX6.1 and NKX2.2 (Fig. 4B). In human islets, *MAFA* expression was positively associated with proinsulin gene expression both in C- and T-allele carriers (Supplementary Material, Table S4). Knocking down *Pdx1* reduced the mRNA expression of *MafA*, *Nkx6.1*, *Nkx2.2*, *Pcsk2*, *Ins1* and *Ins2*. These changes were confirmed at the protein level for all but PCSK2 (Fig. 4C). Knocking down *NeuroD1* reduced the mRNA expression of *Tcf7l2*, *Isl1*, *MafA*, *Pdx1*, *Nkx6.1*, *Nkx2.2*, *Pcsk1*, *Pcsk2*, *Ins1* and *Ins2*. This was,

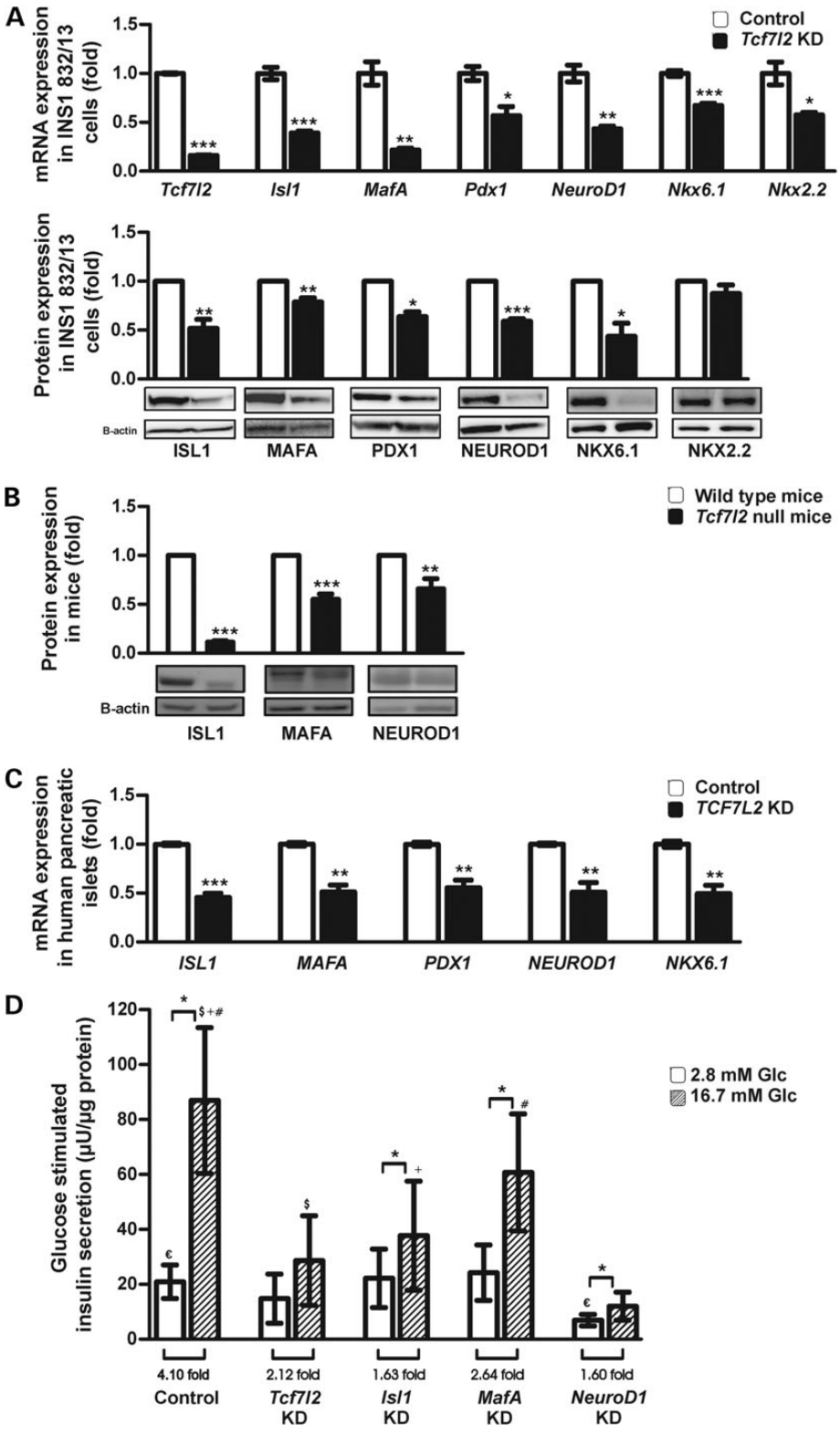

Figure 3. TCF7L2 is regulating proinsulin expression via direct regulation of ISL1 and indirectly via MAFA, NEUROD1 and PDX1. (**A**) mRNA expression measured by QPCR in INS1 cells in *Tcf7l2* silenced versus control cells, ($n = 3$). Protein expression measured by western blot in INS1 cells in *Tcf7l2* silenced versus control cells, ($n = 3$). (**B**) Protein expression measured by western blot in *Tcf7l2* allele-null versus wild-type mice ($n = 6$). (**C**) mRNA expression measured by QPCR in human pancreatic islets in *Tcf7l2* silenced versus control cells, ($n = 3$). (**D**) GSIS in INS1 cells after *Tcf7l2*, *Isl1*, *MafA* and *NeuroD1* silencing ($n = 3$). Data are represented as mean $\pm$ SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ analyzed in (A)–(C) by unpaired Student's *t*-tests. In (D), 2.8 versus 16.7 mM glucose were analyzed by paired Student's *t*-tests (Supplementary Material, Table S1).

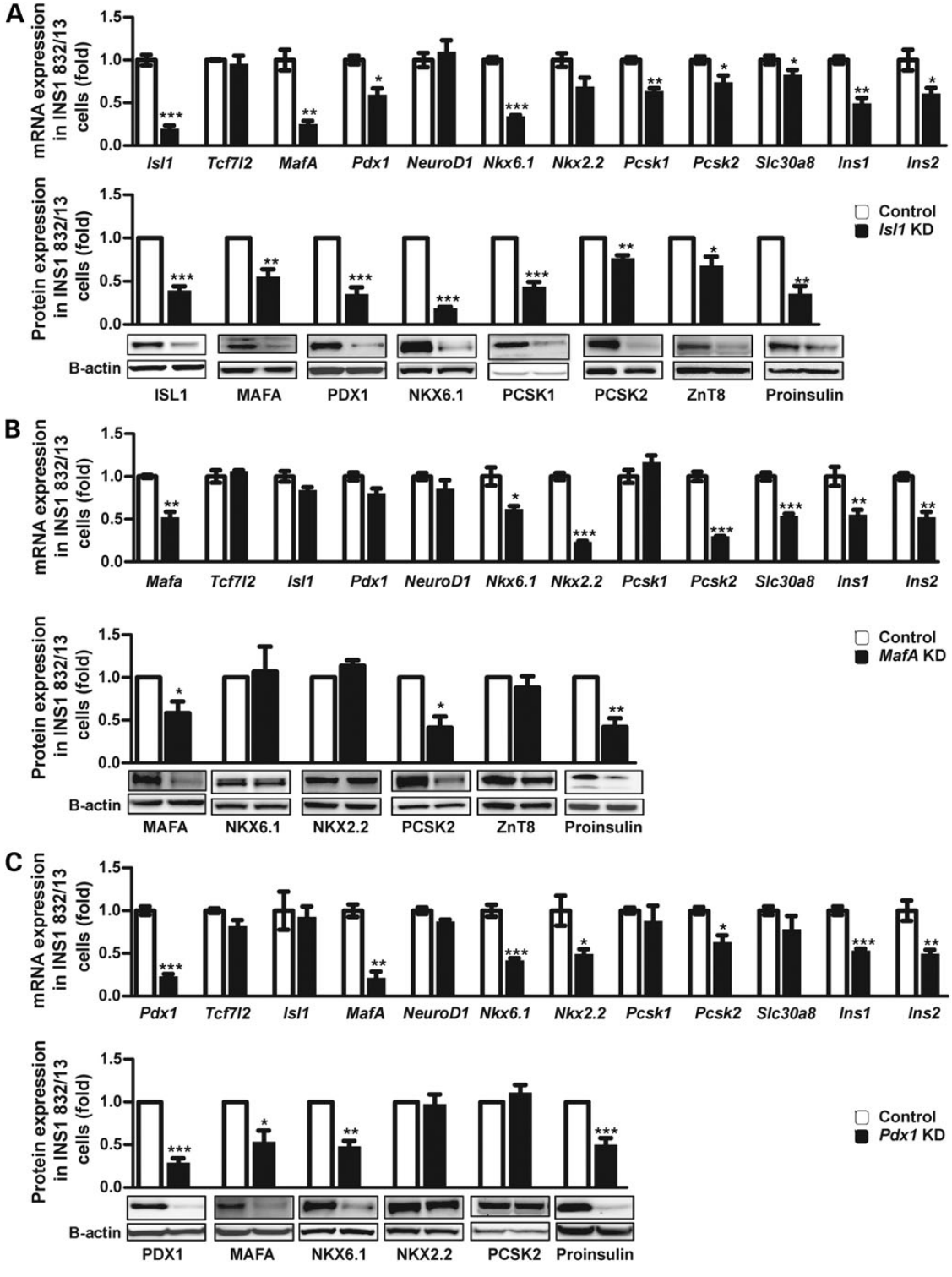

Figure 4. Sequential silencing of TCF7L2-target genes in the proinsulin regulatory cascade. mRNA and protein expression after: (**A**) *Isl1*, (**B**) *MafA*, (**C**) *Pdx1*, (**D**) *NeuroD1* and (**E**) *Nkx6.1* silencing versus controls measured by QPCR and western blot in INS1 cells. Data are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ analyzed by Student's *t*-tests with $n = 3$ (Supplementary Material, Table S1).

however, not reflected by changes in the ISL1, MAFA and PDX1 protein levels (Fig. 4D). Knocking down *Nkx6.1* reduced the mRNA expression of *Tcf7l2*, *Isl1*, *MafA*, *Pdx1*, *Nkx6.1*, *Nkx2.2*, *Pcsk2*, *Ins1* and *Ins2*. At the protein level, we could only confirm the reduction of MAFA and proinsulin (Fig. 4E).

In summary, these data together with previous ChIP-on-Chip results (30) indicate that ISL1 is a direct target of TCF7L2 and that ISL1, in turn regulates proinsulin production and processing via regulation of MAFA, PDX1, NKX6.1, PCSK1 and PCSK2 expression.

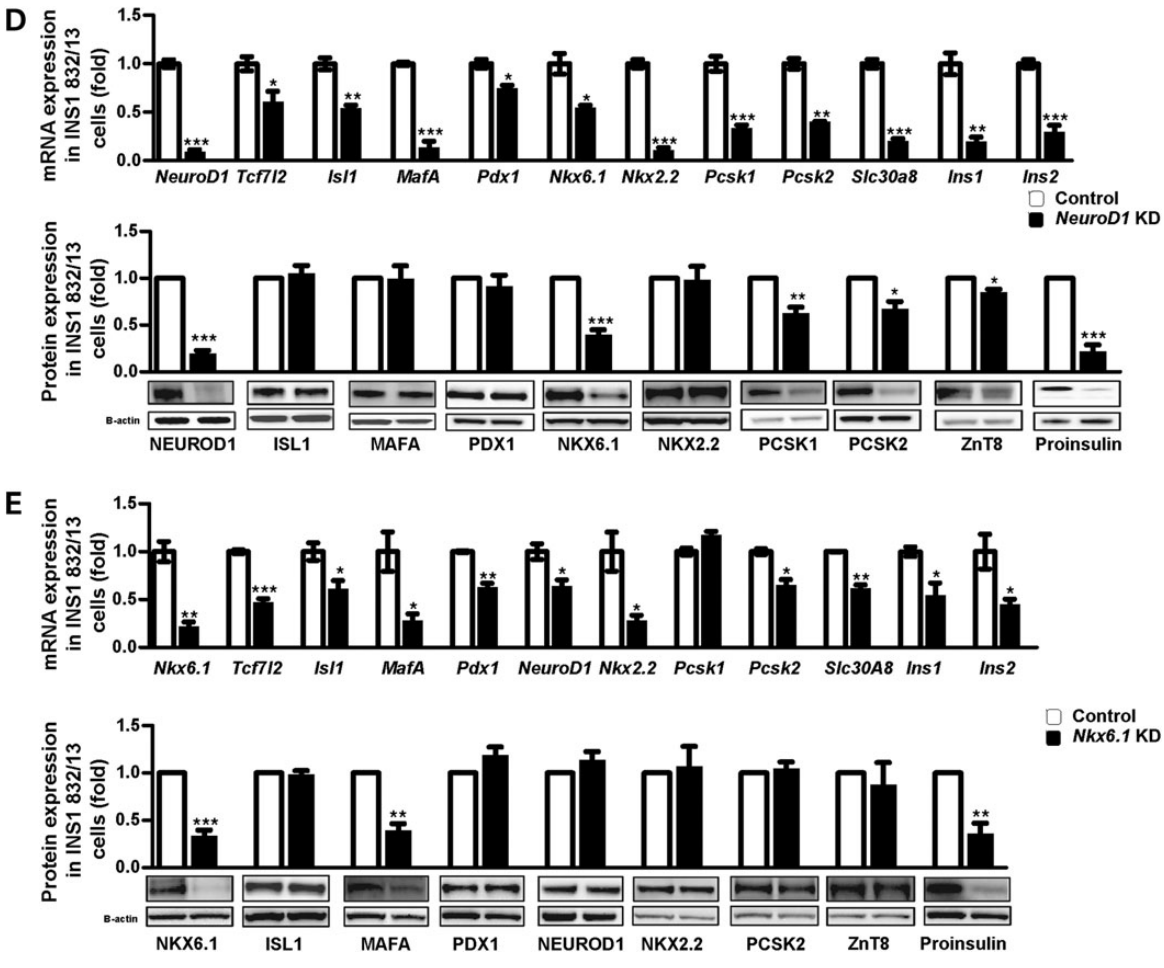

Fig. 4 *Continued*

TCF7L2 is a transcriptional regulator of key genes located in T2D-associated loci

Next, we examined whether silencing of *Tcf7l2* was associated with changes in expression of rat orthologs of putative genes in T2D-associated loci (36,37,41,44–46) (Supplementary Material, Table S2). We observed differential mRNA expression for 23 genes, 17 out of these were replicated using QPCR and 3 at the protein level (i.e. *Slc30a8*, *Ide* and *Bcl11a*) (Figs 2 and 5). Of note, *Tcf7l2* silencing in INS1 cells led to a decreased mRNA expression of *Slc30a8* (Fig. 2A). In pancreata from *Tcf7l2*$^{-/-}$ mice, ZnT8 (*Slc30a8*) expression was reduced by 44% compared with wild-type control mice ($n = 6$, Fig. 2B). This was also replicated in human islets showing a 46% reduction of *SLC30A8* mRNA expression after *TCF7L2* silencing (Fig. 2C). ZnT8 protein expression was also reduced in INS1 cells after *Isl1* and *NeuroD1* silencing (Fig. 4A and D), but not after *Pdx1*, *MafA* and *Nkx6.1* silencing (Fig. 4B, C and E).

Finally, expression (mRNA) of *Grk5*, *Ide*, *Prox1*, *Kcnk16*, *Gipr*, *Adamts9*, *Srebf1*, *Ankrd55*, *Jazf1*, *Ccnd2* and *Tle1* was downregulated, whereas expression of *Vps26a*, *Thada*, *Bcl11a* and *Bcl2* was upregulated after *Tcf7l2* knockdown (Fig. 5; Supplementary Material, Tables S1 and S2).

DISCUSSION

The current report presents a genetic network controlled by TCF7L2 in pancreatic islets, providing a comprehensive view of the molecular mechanisms by which TCF7L2 regulates glucose metabolism. We demonstrate that TCF7L2 plays a central role in coordinating the expression of proinsulin and its subsequent processing to form mature insulin. We also show that TCF7L2 and ISL1 are key regulators of this transcriptional network and provide evidence that they regulate MAFA, PDX1, NEUROD1 and NKX6.1 (Fig. 6). The two prohormone convertases responsible for the processing of proinsulin to mature insulin were also targets of TCF7L2/ISL1 regulation. These functions seem to have been highly conserved during evolution as similar results were obtained in a rat cell line, primary mouse and human islets. In line with a previous report of an overrepresentation of TCF7L2 binding to genes in T2D-associated loci in HCT116 cells (47), we find that TCF7L2 regulates several of these genes also in pancreatic islets. Of note, *SLC30A8* (encoding the zinc transporter ZnT8) is a downstream target of TCF7L2/ISL1 regulation. Zinc is important for the formation of insulin crystals (48), and a common non-synonymous SNP in the zinc transporter *SLC30A8* has been associated with both T2D (40) and reduced proinsulin-to-insulin conversion

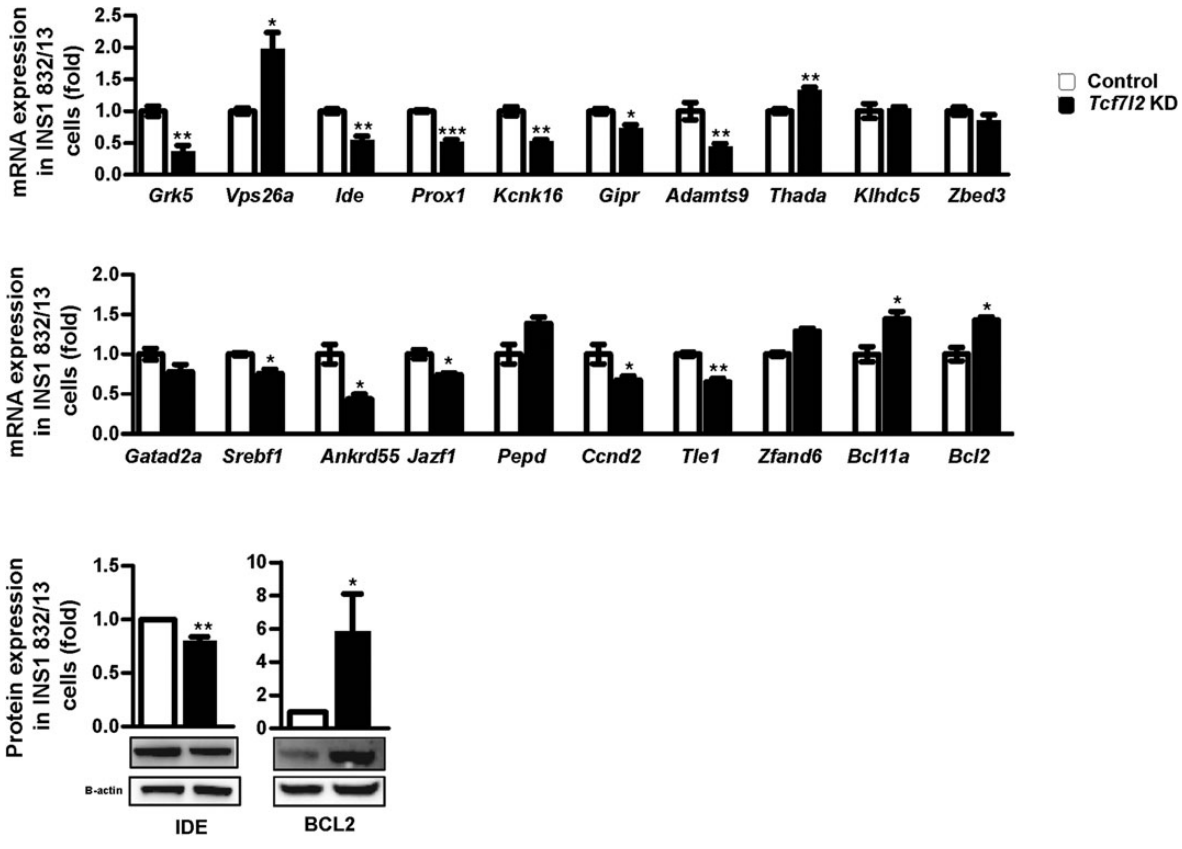

Figure 5. TCF7L2 is a transcriptional regulator of key genes located in T2D-associated loci. mRNA expression measured by QPCR in INS1 cells in *Tcf7l2* silenced versus control cells, ($n = 3$). Protein expression was measured for two genes, i.e. IDE and BCL2, by western blot in INS1 cells in *Tcf7l2* silenced versus controls ($n = 3$). Data are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ analyzed by Student's *t*-tests (Supplementary Material, Table S1).

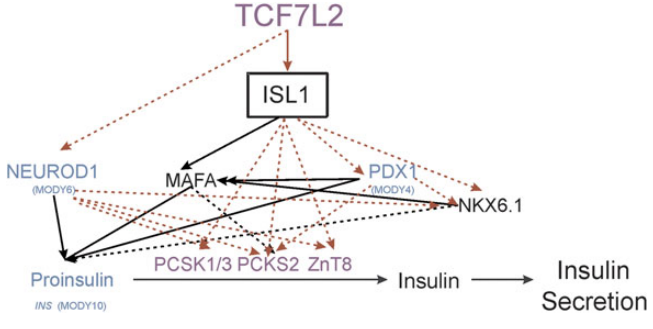

Figure 6. Schematic figure of how TCF7L2 functions as a master regulator of insulin production and processing. Identified TCF7L2-target genes and the downstream regulatory network responsible for its effect on insulin secretion. In rodents, as well as human pancreatic islets, *ISL1* is a direct target of TCF7L2. ISL1 regulates proinsulin production and processing via regulation of PCSK1, PCSK2, SLC30A8, MAFA, PDX1 and NKX6.1. These multiple targets in key pathways may explain why *TCF7L2* has emerged as the gene showing the strongest association with T2D among common variants. Red arrows: molecular links observed for the first time. Black arrows: known molecular links. Direct arrows: direct molecular links. Dash arrows: indirect molecular links. Blue gene symbols: MODY genes. Purple gene symbols: genes associated with T2D and/or (pro)insulin traits.

(15). *SLC30A8* has previously been identified as a TCF7L2-target gene in mice (29), a finding now replicated in humans. Although the mechanisms by which the variant in *SLC30A8* increases risk of T2D are not clear, recent data suggest that reduction in zinc concentrations in the portal vein after β-cell-specific disruption of *Slc30a8* might influence hepatic insulin clearance (49). If confirmed, this would suggest that *TCF7L2* might influence all key steps in (pro)insulin synthesis, processing and clearance.

Transcription of proinsulin in humans is under the control of many glucose responsive TFs that mainly bind a 400 bp conserved region (50,51). Among the most studied TFs are MAFA, PDX1 and NEUROD1, all shown here to be transcriptional targets of TCF7L2/ISL1 regulation. In support of this, ISL1 regulates *MAFA* expression by directly binding to the *MAFA* promoter (52), but its influence on *Pdx1*, *Nkx6.1*, *Pcsk1* and *Pcsk2* expression has not been described to our knowledge. Together with our recent demonstration of direct binding of TCF7L2 to the *Isl1* promoter (30), these data indicate that TCF7L2 together with ISL1 forms a master regulatory circuit influencing both proinsulin expression and processing via regulation of *MafA*, *Pdx1*, *Nkx6.1*, *Pcsk1* and *Pcsk2*. As ISL1 also can bind and influence the expression of other pancreatic hormones like glucagon (53) and somatostatin (54), this indicates that TCF7L2 may also have important regulatory functions in other islet cell types besides the B-cell. Relatively high expression of *TCF7L2* in α-cells has also been reported in sorted human pancreatic islet cells (55). A recent study indicated that NEUROD1 regulates the expression of *Ins1* but not *Ins2* in

primary mouse β cells (56), whereas we observed altered expression of both proinsulin genes and *Nkx6.1* in rat cell line.

TCF7L2 may also regulate other β-cell functions essential for maintaining GSIS. A perturbation of intracellular [Ca^{2+}] was observed in one *Tcf7l2*$^{-/-}$ mouse model, but no major effect on the expression of Ca^{2+} channels (29). Here, we find that although the mRNA levels of several Ca^{2+} channels important for GSIS were reduced in *Tcf7l2* silenced cells, no changes of the corresponding proteins were observed either 72 h or 7 days after *Tcf7l2* knockdown. An effect of TCF7L2 on Ca^{2+} handling in the B-cell should however not be excluded based upon these data as these proteins may have a very long half-life in the cell. An influence on cell viability and regulation of apoptosis are other well-documented effects of TCF7L2 in pancreatic islets (11,30,57). The T2D-associated SNP rs7903146 has also been associated with pancreatic islet morphology, i.e. with reduced islets density, reduction of islet size and an increased α- to β-cell ratio in risk-genotype carriers (31). In the same report, an association between the rs7903146 and GSIS was seen; but not with insulin content per islet equivalent as shown in our study. There may be several explanations for these discrepancies but a large number of high-quality human islets are probably needed to demonstrate a significant reduction in insulin content, as the state of the islets can have a large influence on insulin measurements.

Some recent reports have questioned the dogma that genetic variants in *TCF7L2* exert its major effect on islets (23,58). Boj *et al.* recently reported reduced hepatic glucose production in a liver-specific *Tcf7l2* knockout mouse model (23). We and others have shown an association between the rs7903146 variant and impaired suppression of hepatic glucose production (7,59,60), but it is not easy to disentangle these effects from the effects of a reduction in insulin secretion (61). Surprisingly, they reported no impaired effects on β-cell function in a β-cell-specific *Tcf7l2* knockout mouse (23). This finding is in sharp contrast with previous reports showing reduced β-cell mass, a perturbed incretin response and GSIS with reduced insulin expression (24,27). Although there are no apparent explanations for these discrepant results they could reflect the application of B-cell-specific versus whole-islet knockdown strategies, as *TCF7L2* is also expressed in α-cells (55), or a difference between inducible versus congenital knockdown of *TCF7L2*, as Wnt signaling is important for pancreas development (62). An alternative explanation is a possible compensatory increased expression of other genes taking over the role of TCF7L2 in the β cells, in support is the reported lack of regulation of well-known TCF7L2-target genes, i.e. *Axin2* and *Sp5* (23). *TCF7L2* is expressed in many tissues besides the pancreas and here is no doubt that it has central functions in tissues like the brain (58,63,64), liver (23,65,66), adipose tissue (67,68) and the gut (58,69). However, given the central role of insulin for maintenance of normal glucose tolerance it is unlikely that an extrapancreatic mechanism could exert an effect size of that seen for the rs7903146 in *TCF7L2*.

Interestingly, in human islets we observe a negative association between *TCF7L2* expression and target gene expression in the proinsulin synthesis pathway (Fig. 1D), but also find that silencing total expression of *TCF7L2* leads to reduced proinsulin expression (Fig. 2C). Experimental disruption of TCF7L2 activity has previously been associated with perturbed islet function (8,11,24,29–31) but the risk T-allele is suggested to lead to increased expression of *TCF7L2* (Fig. 1C) (7,22,31,32) and perturbed GSIS in human islets (Fig. 1A) (2,7–12,31). These seemingly contradictory findings might reflect that total expression of *TCF7L2* does not mirror TCF7L2 activity and rather it is the ratio of activating and inhibiting TCF7L2 isoforms that determine the activity and hence target gene regulation (33). The rs7903146 is located near the variable exon 4 (also named 3a), an exon that have particularly high expression in human islets compared with other tissues (4). This exon has also been shown to exhibit regulatory properties, inhibiting TCF7L2-target gene expression in different cell lines (70,71). Loss of exon 4 also promotes hepatic tumorigenicity (72). Future experiments would be warranted to investigate if increased expression of exon 4 containing or other isoforms (67) of TCF7L2 may propagate the risk of T2D conferred by the T-allele of rs7903146.

In summary, in rodents as well as human pancreatic islets, *ISL1* is a direct target of TCF7L2 and ISL1, in turn, regulates proinsulin production and processing via regulation of PCSK1, PCSK2, SLC30A8, MAFA, PDX1 and NKX6.1. Furthermore, TCF7L2 might also influence hepatic clearance of insulin via its effect on SLC30A8. Taken together, the current results provide some explanations for the large impact of *TCF7L2* on the pathogenesis of T2D demonstrating that TCF7L2 is a key regulator of (pro)insulin synthesis, processing and possibly clearance.

MATERIALS AND METHODS

RNA sequencing

Sample preparation was made using Illumina TrueSeq™ RNA sample preparation kit with 1 μg of high-quality total RNA and was sequenced using a paired-end 101 bp protocol on the HiSeq2000 platform (Illumina). For INS1 cells, reads were aligned using Top Hat 2.0.0(73) and Bowtie version: 2.0.0.5 (74) to the rat RGSC3.4 genome assembly. For human islet, 66 samples (all non-diabetic) were sequenced. Sequencing reads were aligned to the human reference genome (hg19) with STAR version 2.3.0e (75) and counted using HTseq v0.5.4 and edgeR (76). The raw sequence reads have been deposited at European Nucleotide Archive (ERA261116) and at GEO (GSE50398) for INS1 and human islets, respectively.

Real-time QPCR and western blot

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen), cDNA was prepared using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific). QPCR was performed using TaqMan assays (Supplementary Material, Table S5). mRNA was measured 48 h (INS1 cells) or 24 h (human islets) after silencing. For human islets, dispersed human islet cells in 804G-conditioned medium-coated dishes (77) were used. Protein (15–80 μg) was separated by SDS–PAGE, transferred to nitrocellulose membranes and detection was made using SuperSignalWestFemto Chemiluminescent Substrate (Thermo Scientific). Protein expression was measured 72 h or 7 days after silencing. Antibodies used can be found in Supplementary Material, Table S5.

Statistical analysis

Statistical analyses were performed in IBM® statistics version 21 (IBM Corporation, Armonk, NY, USA). Student's *t*-test was used when two groups were compared and paired *t*-test was used for analysis of GSIS, GSPS and TIRFM data. $P < 0.05$ was regarded as significant and two-tailed *P*-values were calculated. FDR of 5% was used to correct for multiple comparisons. All results are given as mean ± SEM unless otherwise stated.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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