**TCF7L2 is a master regulator of insulin production and processing**

Yuedan Zhou¹, Soo Young Park², Jing Su³, Kathleen Bailey⁴, Emilia Ottosson-Laakso¹, Liliya Shcherbina⁴, Nikolay Oskolkov¹, Enming Zhang¹, Thomas Thevenin¹, João Fadista¹, Hedvig Bennet¹, Petter Vikman¹, Nils Wierup¹, Malin Fex¹, Johan Rung⁵, Claes Wollheim¹, Marcelo Nobrega⁴, Erik Renström¹, Leif Groop¹, and Ola Hansson¹

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

**Abstract**

Although variants in the T-cell factor 7-like 2 gene (*TCF7L2*) confer the strongest risk of type 2 diabetes (T2D) by presumed effects on islet function, the underlying mechanisms are not well understood. We have identified TCF7L2-target genes and described the regulatory network downstream of TCF7L2 responsible for its effect on insulin secretion in rodents and human pancreatic islets. *ISL1* is a direct target of TCF7L2 and regulates proinsulin production and processing via *MAFA*, *PDX1*, *NKX6.1*, *PCSK1* and *PCSK2* and possibly clearance of proinsulin via *SLC30A8*. 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 in a genotype dependent manner. These multiple targets in key pathways may explain why *TCF7L2* has emerged as the gene showing the strongest association with T2D.

**Corresponding author:**

Ola Hansson
Ola.Hansson@med.lu.se
Lund University
Department of Clinical Science
Endocrinology and Diabetes
Ing 72, Bdg 91, Fl 12
Tel: +46-391228
Fax: +46-391222

Yuedan Zhou
Yuedan.Zhou@med.lu.se
Lund University
Department of Clinical Science
Endocrinology and Diabetes
Ing 72, Bdg 91, Fl 12
Tel: +46-391217
Fax: +46-391222
Introduction

TCF7L2 harbours genetic variants with the strongest effect on T2D risk yet described, i.e. the single nucleotide polymorphism (SNP rs7903146) (Saxena et al., 2012). TCF7L2 is a transcription factor (TF) in the Wnt-signalling pathway and expressed in many tissues including fat, liver and pancreatic islets of Langerhans (Osmark et al., 2009). A majority of variants associated with T2D by GWAS seem to affect islet function (Florez, 2008). In concordance, the risk T-allele of rs7903146 is associated with impaired glucose-stimulated insulin secretion (GSIS) or other secretagogues like GLP-1 (Chang et al., 2010; Loder et al., 2008; Lyssenko et al., 2007; Rosengren et al., 2012; Saxena et al., 2006; Shu et al., 2009; Shu et al., 2008). Risk T-allele carriers are further characterized by an elevated plasma proinsulin level and an increased proinsulin/insulin ratio suggestive of perturbed proinsulin processing (Dahlgren et al., 2007; Dupuis et al., 2010; Gonzalez-Sanchez et al., 2008; Kirchhoff et al., 2008; Loos et al., 2007; McCaffery et al., 2011; Silbernagel et al., 2011; Stolerman et al., 2009; Strawbridge et al., 2011). 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 (Gaulton et al., 2010). Several mouse models of Tcf7l2 have been investigated (Boj et al., 2012; da Silva Xavier et al., 2012; Korinek et al., 1998; Savic et al., 2011b; Takamoto et al., 2013; Yang et al., 2012), 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 (da Silva Xavier et al., 2009; Shu et al., 2008; Zhou et al., 2012).

Paradoxically, carriers of the risk T-allele show increased expression of TCF7L2 in human pancreatic islets (Le Bacquer et al., 2012; Lyssenko et al., 2007). The T-allele locus displays much stronger transcriptional activity (Gaulton et al., 2010; Stitzel et al., 2010). 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 (Hansson et al., 2010). However, the target genes of TCF7L2 in pancreatic islets or β-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* (Lyssenko et al., 2007) and *in vitro* (Le Bacquer et al., 2012; Rosengren et al., 2012) 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, Figure 1A and Supplemental Table S3). We also show reduced insulin content (27%) in risk T-allele carriers (n=81, Figure 1B), and that TCF7L2 mRNA expression is 16% higher than C-allele carriers (n=66; RNA-seq, Figure 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, Figure 1D, Table S4).

Silencing Tcf7l2 in a rat insulinoma cell line (INS1 832/13) using siRNA targeting the invariable exon 1, resulted in 84% knock-down of Tcf7l2 mRNA, 90% reduction of TCF7L2 activity and a reduced expression of the well-known TCF7L2 target genes *Axin2* and *Ccnd1* (Figure S1A-B)(Zhou et al., 2012). 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, Figure 1E) and a perturbed glucose-stimulated proinsulin secretion (GSPS) (p<0.05, Figure 1F). Proinsulin/insulin ratio was higher under glucose stimulation compared to basal condition in Tcf7l2 silenced cells (p<0.05, Figure 1G) suggesting perturbed proinsulin processing. Immunohistochemistry (IHC) analysis of Tcf7l2 silenced INS1 cells showed reduced staining intensity for insulin (p<0.00001) and VAMP2 (p<0.00000001), a marker of insulin-containing granules, in Tcf7l2 silenced cells (Figure 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, Figure 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 β-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 count per gene); 1680
genes were up-regulated and 1885 genes were down-regulated in Tcf7l2 silenced cells (5% 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 (Hamid et al., 2004), but this was not replicated (Qi et al., 2007) or supported by recent GWAS findings (Cho et al., 2012; Dupuis et al., 2010; Kooner et al., 2011; Saxena et al., 2007; Scott et al., 2007; Sladek et al., 2007; Voight et al., 2010; Zeggini et al., 2007) 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 (Table 1). In addition, genes located proximal to SNPs with replicated associations to T2D and/or plasma proinsulin were also selected for further analysis (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 (Table S1).

Tcf7l2 is a key regulator of proinsulin production and processing

Twelve analyzed genes involved in proinsulin synthesis were down-regulated in Tcf7l2 silenced cells, including proinsulin genes Ins1 and Ins2 (Table 1 and Figure 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 (Figure 2B). We next replicated this finding in primary cells by measuring proinsulin protein expression in a Tcf7l2 homozygous null mouse model (Tcf7l2-/-)(Savic et al., 2011b). Notably proinsulin expression was reduced by 85% (n=6) in pancreas from Tcf7l2-/- mice (postnatal day 0; P0) compared to wild type mice (Figure 2C). Given differences in the insulin gene between rodents and humans(Soares et al., 1985), we also replicated the findings in human pancreatic islets showing 49% reduced proinsulin mRNA expression after TCF7L2 silencing (n=5, Figure 2D).

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 (Figure 2A-B). No significant change in PCSK1 and PCSK2 protein expression was observed in the Tcf7l2<sup>−/−</sup> mouse pancreas (Figure 2C). In TCF7L2 silenced human pancreatic islets, PCSK1 and PCSK2 mRNA expression was reduced by 36% and 49% respectively (Figure 2D).

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

Of the 11 genes categorized as involved in the exocytic machinery, mRNA expression of Cacna1a, Cana1c, Cacna1d and Cacnb3 was reduced in Tcf7l2 silenced cells (Table 1 and Supplemental Figure S1C). However, this was not confirmed at the protein level for Cav2.1, Cav1.2 or Cav1.3, either 72 hours, or 7 days after Tcf7l2 silencing (Figure S1D-E). RNA expression of 2 out of the 5 genes involved in glucose sensing (Glud1 and Slc2a2/Slut2) was reduced at the mRNA level in Tcf7l2 silenced cells (Table 1), and GLUD1 protein expression was measured but no change was observed (Figure S1F).

**Tcf7l2 is regulating proinsulin expression directly via Isl1, Ins1 and Ins2 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 (Figure 3A). The reduction of ISL1 and NEUROD1 was further confirmed using IHC (Figure S1G-H). These results were confirmed in Tcf7l2<sup>−/−</sup> mice, with protein levels reduced relative to wild type mice for ISL1, MAFA and NEUROD1 (Figure 3B). Likewise, after TCF7L2 silencing in human islets, expression of most transcripts was down-regulated, including ISL1, MAFA, PDX1, NEUROD1 and NKX6.1 (Figure 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 (Table S4). The disruption of Tcf7l2 resulted in a
marked reduction in GSIS compared with controls 1.93 (p>0.05) vs. 4.10 fold (p<0.05) increases (Figure 3D). The same was observed after disruption of Isl1, MafA and NeuroD1 - (Figure 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 (Figure 4A). No effect of Isl1 knock-down was observed on Tcf7l2 or NeuroD1 mRNA expression (Figure 4A). Previously we have analysed chromatin immunoprecipitation on tiling array (ChIP-on-Chip) using a TCF7L2 antibody identifying both the Isl1 and Ins1 promoters as directly bound by TCF7L2 (Zhou et al., 2012). 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, NNX6.1, INS and SLC30A8 but in T-allele carriers only with PDX1 and NNX6.1 (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 (Figure 4B). In human islets, MAFA expression was positively associated with proinsulin gene expression both in C- and T-allele carriers (Table S4). Knocking down Pdx1 reduced the mRNA expression of MafA, Nkx6.1, Nkx2.2, Pcsk1, Pcsk2, Ins1 and Ins2. These changes were confirmed at the protein level for all but PCSK2 (Figure 4C). Knocking down NeuroD1 reduced the mRNA expression of Tcf7l2, Isl1, MafA, Pdx1, Nkx6.1, Nkx2.2, Pcsk1, Pcsk2, Ins1 and Ins2. This was, however, not reflected by changes in the ISL1, MAFA and PDX1 protein levels (Figure 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 (Figure 4E).

In summary, these data together with previous ChIP-on-Chip results (Zhou et al., 2012) indicate that ISL1 is a direct target of TCF7L2 and that ISL1, in turn regulates proinsulin production and processing via regulation of MAFA, PDX1, NNX6.1, PCSK1 and PCSK2 expression.

**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 orthologues of putative genes in T2D–associated loci (Cho et al., 2012; de Miguel-Yanes et al., 2011; Kooner et al., 2011; Morris et al., 2012; Saxena et al., 2012; Voight et al., 2010)(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)-(Figure 2 and 5). Of note, Tcf7l2 silencing in INS1 cells led to a decreased mRNA expression of Slc30a8 (Figure 2A). In pancreata from Tcf7l2<sup>-/-</sup> mice, ZnT8 (Slc30a8) expression was reduced by 44% compared to wild type control mice (n=6, Figure 2C). This was also replicated in human islets showing a 46% reduction of SLC30A8 mRNA expression after TCF7L2 silencing (Figure 2D). ZnT8 protein expression was also reduced in INS1 cells after Isl1 and NeuroD1 silencing (Figure 4A-D), but not after Pdx1, MafA and Nkx6.1 silencing (Figure 4B, C and E).

Finally, expression (mRNA) of Grk5, Ide, Prox1, Kcnk16, Gipr, Adamts9, Srebf1, Ankrd55, Jazf1, Ccnd2 and Tle1 was down-regulated whereas expression of Vps26a, Thada, Bcl11a and Bcl2 was up-regulated after Tcf7l2 knock-down (Table S2). In addition, the BCL11A protein expression was markedly up-regulated after disruption of Isl1 and NeuroD1 (Figure 4A and D).
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 (Figure 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. TCF7L2 is also regulates several genes in T2D-associated loci. Of note, \textit{SLC30A8} (encoding the zinc transporter ZnT8) is a downstream target of TCF7L2/ISL1-regulation. Zinc is important for the formation of insulin crystals (Scott, 1934), and a common non-synonymous SNP in the zinc transporter \textit{SLC30A8} has been associated with both T2D (Sladek et al., 2007) and reduced proinsulin-to-insulin conversion (Kirchhoff et al., 2008). \textit{SLC30A8} has previously been identified as a TCF7L2-target gene in mice (da Silva Xavier et al., 2009), a finding now replicated in humans. Although the mechanisms by which the variant in \textit{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 \textit{Slc30a8} might influence hepatic insulin clearance (Tamaki et al., 2013). 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 base pair conserved region (Fu et al., 2013; Hay and Docherty, 2006). 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 \textit{MAFA} expression by directly binding to the \textit{MAFA} promoter (Du et al., 2009), but its influence on \textit{Pdx1, Nkx6.1, Pcsk1} and \textit{Pcsk2} expression has not been described to our knowledge. Together with our recent demonstration of direct binding of TCF7L2 to the \textit{Isl1} promoter (Zhou et al., 2012), these data indicate that TCF7L2 together with ISL1 forms a master regulatory circuit influencing both proinsulin expression and processing via regulation of \textit{MafA, Pdx1,}
Nkx6.1, Pcsk1 and Pcsk2. As ISL1 also can bind and influence the expression of other pancreatic hormones like glucagon (Wang and Drucker, 1995) and somatostatin (Leonard et al., 1992), this indicates that TCF7L2 may also have important regulatory functions in other islet cell types besides the β-cell. Relatively high expression of TCF7L2 in α-cells has also been reported in sorted human pancreatic islet cells (Kirkpatrick et al., 2010). A recent study indicated that NEUROD1 regulates the expression of Ins1 but not Ins2 in primary mouse β-cells (Gu et al., 2010), while we observed altered expression of both proinsulin genes as well as 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 (da Silva Xavier et al., 2009). 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 hours or 7 days after Tcf7l2 knock-down. An effect of TCF7L2 on Ca^{2+} handling in the β-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 (Le Bacquer et al., 2011; Shu et al., 2008; Zhou et al., 2012). 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 (Le Bacquer et al., 2012). In the same report, an association between the rs7903146 variant and impaired suppression of hepatic glucose production (Lyssenko et al., 2007; Pilgaard et al., 2009; Wegner et al., 2008), but it is not easy to disentangle these effects from the effects of a reduction in insulin secretion (Faerch et al., 2013). Surprisingly, they reported no impaired effects on β-cell function in a β-cell specific Tcf7l2 knockout mouse (Boj et al., 2012). This
finding is in sharp contrast with previous reports showing reduced β-cell mass, a perturbed incretin response and GSIS with reduced insulin expression (da Silva Xavier et al., 2012; Takamoto et al., 2013). Although there are no apparent explanations for these discrepant results they could reflect the application of β-cell specific versus whole-islet knock-down strategies, as TCF7L2 is also expressed in α-cells (Kirkpatrick et al., 2010), or a difference between inducible versus congenital knock-down of TCF7L2, as Wnt-signaling is important for pancreas development (Murtaugh, 2008). 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 (Boj et al., 2012). TCF7L2 is expressed in many tissues besides the pancreas and there is no doubt that it has central functions in tissues like the brain (Lee et al., 2009; Savic et al., 2011a; Shao et al., 2013), liver (Boj et al., 2012; Ip et al., 2012; Oh et al., 2012), adipose tissue (Mondal et al., 2010; Prokunina-Olsson et al., 2009) and the gut (Kaminska et al., 2012; Shao et al., 2013). 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 (Figure 1D), but also find that silencing total expression of TCF7L2 leads to reduced proinsulin expression (Figure 2C). Experimental disruption of TCF7L2 activity has previously been associated with perturbed islet function (da Silva Xavier et al., 2009; da Silva Xavier et al., 2012; Le Bacquer et al., 2012; Shu et al., 2009; Shu et al., 2008; Zhou et al., 2012) but the risk T-allele is suggested to lead to increased expression of TCF7L2 (Figure 1C)(Gaulton et al., 2010; Le Bacquer et al., 2012; Lyssenko et al., 2007; Stitzel et al., 2010) and perturbed GSIS in human islets (Figure 1A)(Chang et al., 2010; Le Bacquer et al., 2012; Loder et al., 2008; Lyssenko et al., 2007; Rosengren et al., 2012; Saxena et al., 2006; Shu et al., 2009; Shu et al., 2008). These seemingly contradictory findings might reflect that total expression of TCF7L2 do 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 (Hansson et al., 2010).

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.
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(Kim et al., 2013) and Bowtie version: 2.0.0.5 (Langmead and Salzberg, 2012) 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 (Dobin et al., 2013) and counted using HTseq v0.5.4 and edgeR (Robinson et al., 2010). The raw sequence reads have been deposited at European Nucleotide Archive (ERA261116) and at GEO (GSE50398) for INS1 and human islets, respectively.

Real-time quantitative PCR 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 (Table S5). mRNA were measured 48 hours (INS1 cells) or 24 hours (human islets) after silencing. For human islets, dispersed human islet cells in 804G-conditioned medium coated dishes(Hammar et al., 2004) 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 hours or 7 days after silencing. Antibodies used can be found in Table S5.

Statistical Analysis

Statistical analyses were performed in IBM® statistics version 21 (IBM Corporation, Armonk, NY). 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. False discovery rate (FDR) of 5% was used to correct for multiple comparisons. All results are given as mean ± standard error of the mean (SEM) unless otherwise stated.

Data Access

ArrayExpress Accession: E-MTAB-2034
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Disclosure declaration

We hereby declare that none of the authors have a financial interest related to this work.
Reference


preproinsulin I gene is a functional retroposon. Molecular and cellular biology 5, 2090-2103.


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

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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-t tests; N = 4 (RNAseq), N = 3 (QPCR).

* very low expression, **Pdx1 was not aligned in the RNA-seq analysis.
Figure Legend

Figure 1. The type 2 diabetes associated SNP rs7903146 and TCF7L2 expression influences insulin synthesis and secretion.

(A) Glucose-stimulated insulin secretion 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) Glucose-stimulated insulin secretion in INS1 cells after Tcf7l2 silencing.
(F) Glucose-stimulated proinsulin secretion in INS1 cells after Tcf7l2 silencing.
(G) Proinsulin-to-insulin ratio in INS1 cells after Tcf7l2 silencing.
(H) Immunohistochemistry staining of insulin and Vamp2 in INS1 cells after Tcf7l2 silencing.
(I) Amount of insulin 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, *** = p < 0.001, (Table S1 and S4).

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).
(B) Protein expression measured by Western blot in Tcf7l2 silenced- versus control cells, (n = 3).
(C) mRNA expression measured by QPCR in human pancreatic islets in Tcf7l2 silenced- versus control cells, (n = 3 – 5). Data are represented as mean ± SEM. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 analyzed by Student’s t-tests, (Table S1).

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).
(B) Protein expression measured by Western blot in Tcf7l2 silenced- versus control cells, (n = 3).
(C) mRNA expression measured by QPCR in human pancreatic islets in Tcf7l2 silenced- versus control cells, (n = 3).
(D) Glucose-stimulated insulin secretion in INS1 cells after Tcf7l2, Isl1, MafA and NeuroD1 silencing, (n = 3). Data are represented as mean ± SEM. * = p < 0.05, ** = p < 0.01, *** = 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 (Table S1).

Figure 4. Sequential silencing of TCF7L2 target genes in the proinsulin regulatory cascade.

mRNA and protein expression after: Isl1 (A), MafA (B), Pdx1 (C), NeuroD1 (D) and Nkx6.1 (E) silencing versus controls measured by QPCR and Western blot in INS1 cells. Data are represented as mean ± SEM. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 analyzed by Student’s t-tests with n = 3, (Table S1).
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, *** = p < 0.001 analyzed by Student’s t-tests, (Table S1).

Figure 6. Schematic figure of how TCF7L2 functions as a master regulator of insulin production and processing
Schematic figure showing 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.
The diagram illustrates the relationships between various genes and proteins involved in insulin secretion and diabetes. Key genes include TCF7L2, ISL1, NEUROD1, MAFA, PDX1, NFX6.1, PCSK1/3, PCKS2, ZnT8, and INS (MODY10). The color coding indicates the status of previously reported (black) and not reported (red) associations. The dashed lines represent indirect links. The diagram emphasizes the complex network of interactions that regulate insulin secretion and its role in diabetes.