Differential Transcriptional and Posttranslational Transcription Factor 7-Like 2 Regulation Among Nondiabetic Individuals and Type 2 Diabetic Patients

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Human genetic studies have revealed that the T minor allele of single nucleotide polymorphism rs7903146 in the transcription factor 7-like 2 (*TCF7L2*) gene is strongly associated with an increased risk of diabetes by 30%–40%. Molecular and clinical studies are of great importance for understanding how this unique variation in *TCF7L2* influences type 2 diabetes (T2D) onset and progression. At the molecular level, some studies have been performed in diabetic mice and pancreatic islets from healthy human donors. Whereas *TCF7L2* mRNA levels are up-regulated in islets, protein levels are down-regulated. We performed studies on *TCF7L2* splicing, mRNA expression, and protein levels in immortalized human lymphocytes from nondiabetic individuals and T2D patients carrying the *C/C* or the at-risk *T/T* genotype. Our results show differential expression of *TCF7L2* splice variants between nondiabetic and T2D patients carrying the at-risk genotype, as well as differences in protein levels. Therefore, we investigated the regulation of splice variants, and our results propose that splicing of exon 4 is under control of the serine-arginine–rich factor transformer 2 β (*TRA2B*). Finally, we studied the endoplasmic reticulum stress pathways, looking for a posttranslational explanation. We saw a shift in the activation of these pathways between nondiabetic individuals and T2D patients carrying the at-risk genotype. These results suggest that, in human immortalized lymphocytes carrying the at-risk *T/T* genotype, first the differential expression of *TCF7L2* splice variants implies a regulation, at least for exon 4, by *TRA2B* and second, the differential protein levels between both *T/T* carriers point to a different activation of endoplasmic reticulum stress pathways. **(*Molecular Endocrinology* 28: 1558–1570, 2014)**

Type 2 diabetes (T2D) prevalence has reached epidemic proportions throughout the world, and it is the most common metabolic disorder in Western countries associated with obesity (1). T2D is a complex disease and involves the interplay between environmental risk factors and predisposing genetic backgrounds.

To date, several genome-wide association studies have identified the genes most frequently associated with T2D (2). One of the most relevant genes among them is the transcription factor 7-like 2 (*TCF7L2*, previously known as T-cell factor [TCF] 4), in which a single nucleotide polymorphism (SNP), rs7903146, in intron 4, consisting of a change from a cytosine (C) to a thymine (T) nucleotide, has been strongly correlated with an increased risk of developing T2D (3). TCF7L2 is a ubiquitous protein that belongs to a family of TCF/lymphoid enhancer factor

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Abbreviations: ASK1, apoptosis signal–regulating kinase; ATF6, activating transcription factor 6; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum–associated degradation; ESE, exonic splicing enhancer; HNF4α, hepatocyte nuclear factor 4α; hnRNP, heterogeneous nuclear ribonucleoprotein; IRE1, inositol-requiring protein 1; ISE, intronic splicing enhancer; JNK, c-Jun NH_2-terminal kinase; qRT-PCR, quantitative real-time PCR; PERK, protein kinase receptor–like endoplasmic reticulum kinase; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; SR, serine- arginine–rich; T2D, type 2 diabetes; TCF, T-cell factor; TRAF2, TNF receptor-associated factor 2; UPR, unfolded protein response; XBP1, X-box binding protein 1.

transcription factors. The T2D risk–associated *T/T* genotype is present approximately in 7%–10% of the nondiabetic population, whereas its prevalence increases up to 14%–20% of the total diabetic population (3–5).

The human *TCF7L2* gene is encoded by 17 identified exons, 5 of which are subject to alternative splicing (exons 4, 13, 14, 15, and 16) (6). To date, extensive work has been done to evaluate the splicing diversity and expression of *TCF7L2* in a broad range of human and mouse tissues, including not only pancreatic islets but also liver, gut, brain, and adipose tissues among others. Prokunina-Olsson et al (7) analyzed the *TCF7L2* splicing isoform pattern in different types of human tissue (pancreas, pancreatic islets, colon, liver, monocytes, skeletal muscle, subcutaneous adipose tissue, and lymphoblastoid cell lines) and observed a tissue-specific pattern of alternative splicing. In addition, Prokunina-Olsson and Hall (8) further characterized the *TCF7L2* 3′ end splicing variants in different human tissues, observing that these transcripts may share neuroendocrine functions important for brain, gut, and pancreatic islets. Recently Shao et al (9) found that in gut endocrine L-cell lines, *TCF7L2* controls transcription of the proglucagon gene (*gcg*). They generated transgenic mice that express dominant-negative *TCF7L2* specifically in *gcg*-expressing cells, concluding that *TCF7L2* and Wnt signaling control gut and brain *gcg* expression and glucose homeostasis (9). In 2012, Oh et al (10) observed that the expression of medium and short isoforms of *TCF7L2* in liver was greatly diminished in both diet-induced and genetic mouse models of insulin resistance. They found that nuclear isoforms of *TCF7L2* are capable of binding to promoters of gluconeogenic genes, suggesting a crucial role for *TCF7L2* in hepatic glucose metabolism (10). Recently, Neve et al (11) addressed the impact of *TCF7L2* isoforms on hepatocyte nuclear factor 4α (HNF4α) and on the regulation of gluconeogenic genes in humans. They described different expression levels of the *TCF7L2* 3′ end splicing variants in HepG2 cells and in liver, suggesting that these transcripts play a role in the pathophysiology of type 2 diabetes. Moreover, they showed that 2 *TCF7L2* splicing variants, lacking exon 4 and including extranucleotides of exon 7 but with different carboxyl terminal exons, interact with HNF4α (11). Some of these studies have revealed a splicing similarity between pancreatic islets and blood lymphocytes, although *TCF7L2* expression is lower in the latter tissue (7, 12).

Focusing on pancreatic islets, biological studies have clearly demonstrated the role of *TCF7L2* in insulin secretion and in the survival of isolated pancreatic islets (13, 14). However, the molecular mechanisms describing how the genetic variation of *TCF7L2* leads to altered biological function are still largely controversial. *TCF7L2* SNPs could lead to impaired *TCF7L2* function and increased diabetes risk by changing the distribution of mRNA splice variants (7, 12, 13, 15, 16).

Splicing is the process by which exons are joined together by the removal of introns to produce a mature mRNA, which can then be further translated. Splicing is usually constitutive. However, alternative splicing has also been observed, in which the exons may be combined in some other way, introducing new coding information into mRNAs. Alternative splicing affects the vast majority of human genes (>90%) (17). Consequently, multiple mRNAs and protein isoforms are produced from a relatively small number of genes, such as *TCF7L2*, thus becoming one of the main sources of protein diversity (18, 19). Because of the relevance of alternative splicing, this molecular mechanism is highly regulated by both *cis*-regulatory sequences and *trans*-acting factors (Supplemental Figure 1C). *Cis*-regulatory sequences include exonic splicing enhancers (ESEs), exonic splicing silencers, intronic splicing enhancers(ISEs), and intronic splicing silencers, depending on their locations and on how they affect the selection of a splice site. On the other hand, *trans*-acting factors act through binding to splicing enhancers and silencers and include members of well-characterized serine-arginine–rich (SR) proteins (20) and heterogeneous nuclear ribonucleoproteins (hnRNPs (21), as well as tissue-specific factors such as FOX (22), NOVA (23), nPTB, and PTB (24). The interplay between the different components of the spliceosome specifically defines the alternative splicing of each exon.

Once the splicing is over, the mRNA must be translated into protein, but the newly produced proteins are not immediately functional. To become properly functional, they have to enter the endoplasmic reticulum (ER), where they are folded and assembled. To ascertain fidelity in protein folding, cells regulate the protein-folding capacity in the ER according to their needs. The ER responds to unfolded proteins in its lumen (ER stress) by activating intracellular signal transduction pathways that coordinate adaptive and apoptotic responses, collectively termed the unfolded protein response (UPR). The most ER-proximal regulators of the UPR consist of a set of transmembrane ER-resident proteins, including inositol-requiring protein 1 (IRE1), protein kinase receptor–like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). These proteins bear domains protruding into the ER lumen, which sense ER stress, coupled to cytosolic effector domains. Together, these 3 mechanistically distinct branches of the UPR regulate the expression of numerous genes that maintain homeostasis in the ER or induce apoptosis, if ER stress re-

mains unmitigated. There is accumulative evidence implicating prolonged ER stress in the development and progression of many diseases, including T2D (25, 26).

Transcription and translation of the *TCF7L2* gene are oppositely regulated in T2D (14, 27). The possible explanation for this opposing regulation at RNA and protein levels is that increased mRNA expression reflects several *TCF7L2* transcripts. On the translational level, these transcripts could encode fewer or different protein isoforms with different effects on target cells. Therefore, in the present study we characterized the expression of the different splice variants and protein levels of TCF7L2 in human immortalized lymphocytes, distinguishing between nondiabetic and T2D patients carrying the *C/C* or the at-risk *T/T* genotype.

Materials and Methods

Genetic analysis and manipulations were performed after informed written consent was received from the individuals in the study, upon approval by the institutional ethics committee.

Study samples and clinical characteristics

Caucasian Spanish participants recruited were 110 nondiabetic individuals and 110 T2D patients. SNP rs7903146 was genotyped in all participants to find the carriers of the *T/T* at-risk genotype. Only 7 of 110 nondiabetic subjects were homozygous *T/T* carriers, consistent with results of previous epidemiological studies, in which 7%–10% of the population carried the at-risk genotype. After genotyping, 28 subjects were selected (14 nondiabetic control subjects and 14 T2D patients, each group with 7 individuals homozygous for the *C* allele or the *T* allele), matched by body mass index and age and sex distribution (Table 1). Diabetes was diagnosed by following the American Diabetes Association recommendations (28, 29), and none of the control subjects had diabetes or prediabetes.

Study group definition

To perform the experiments, we delimited 4 different groups based on the SNP rs7903146 genotype and the presence or absence of diabetes. Thus, we designated the nondiabetic individuals carrying the nonrisk *C/C* genotype as the control group, because this group does not carry the at-risk *T/T* genotype or the diabetic background. Furthermore, we designated the T2D patients carrying the at-risk *T/T* genotype as our study group, because they present both the risk genotype and diabetes. Finally, we decided to include another 2 groups, to help us discern between the diabetic background (T2D patients carrying the nonrisk *C/C* genotype) and the genetic background (nondiabetic individuals carrying the at-risk *T/T* genotype).

Table 1. Brief Summary of Clinical and Genetic Features of the Patient Cohort

	Male/ Female	Age, y		Body Mass Index, kg/m²	
		Mean	SEM	Mean	SEM
Non-T2D *C/C*	5/2	52.29	4.65	24.96	1.01
Non-T2D *T/T*	5/2	59.29	3.40	27.55	0.68
T2D *C/C*	5/2	58.86	3.60	27.25	1.44
T2D *T/T*	5/2	61.14	3.38	27.25	1.68

Immortalization of human B lymphocytes and cell culture

Lymphocytes were immortalized by the Biobank of the Institut d'investigacions Biomèdiques August Pi i Sunyer (Barcelona, Spain). In brief, lymphocytes were isolated from whole blood by gradient centrifugation with Ficoll, and immortalization was induced by Epstein-Barr virus infection. Immortalized lymphocyte cultures were maintained with RPMI 1640 medium without glucose and glutamine (Biosera) supplemented with 10% fetal bovine serum, penicillin-streptomycin, and L-glutamine (HyClone).

Stimulated cell culture

Immortalized lymphocytes were cultured under nonphysiological conditions. Insulin was added to 1.5 million immortalized lymphocytes at a concentration of 100 nM, and concurrently we cultured the cells with an elevated glucose concentration (25 mM). In both experiments, cells were incubated at 37°C overnight.

Cell transfection

Immortalized lymphocytes were seeded at a density of 2×10^5 cells/well and split onto nontreated 24-well plates (Costar). Cells were transfected with 100 nM ON-TARGETplus human *TRA2B* small interfering RNA (siRNA) or ON-TARGETplus non-targeting pool siRNA (Dharmacon) using Metafectene Pro (Biontex Laboratories GmbH) as the transfection reagent, following the manufacturer's instructions.

RNA isolation

Total RNA was extracted according to the manufacturer's instructions using an RNeasy Mini plus kit (Qiagen). RNA quantity and purity were determined by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), and the RNA was electrophoresed in a 1.5% agarose gel (1 × Tris borate–EDTA [TBE] buffer in RNase-free water) to exclude degradation of RNA and contamination with DNA.

cDNA synthesis

The reverse transcription reaction was performed using a commercially available set (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems). cDNA was prepared from 1 μg of mRNA, with random hexamer primers, according to the manufacturer's instructions. The resulting cDNA was diluted to a final concentration of 5 ng/μL and stored at −20°C.

Primers and amplicon design

To avoid amplification of contaminating genomic DNA, at least one of the primers was designed over exon-exon junctions, and the forward primer was designed to hybridize to one exon and the reverse primer to another exon, producing an amplicon

with an optimal size between 85 and 150 bp (Supplemental Table 1).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems) with a 384-well reaction plate. All amplifications were carried out at least in duplicate. The reaction was performed using SYBR Premix Ex Taq (Tli RNase H Plus) (Takara-Clontech) in a 10-μL total volume. In brief, 2 μL of diluted cDNA template and 8 μL of reaction mixture consisting of 5 μL of SYBR Premix Ex Taq (2×) and 0.2 μL of ROX reference dye (50×), 0.5 μL of each forward and reverse primer (10 μM), and 1.8 μL of nuclease-free water were run using a standard cycling program as follows: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds. Dissociation curve analysis of the PCR amplicons was performed at the end of amplification to verify single product amplification.

The relative quantification of gene expression in each sample was analyzed using SDS software version 2.4 (Applied Biosystems). The C_t value for each sample was measured in duplicate, and the average was normalized against the endogenous control β-actin to determine the ΔC_t value. The ΔC_t values were then standardized against the calibrator's ΔC_t values (*C/C* nondiabetic) to yield the $\Delta\Delta C_t$, and the relative quantification was calculated as $2^{-\Delta\Delta C_t}$ (30).

The analyzed splice variants of *TCF7L2* and *TRA2B* were intranormalized with their own total mRNA expression.

Apoptosis measurement

The caspase activity was measured by the commercial Caspase-Glo 3/7 assay (Promega) following the manufacturer's instructions. We cultured 15 000 cells/well in 100 μL of RPMI 1640 medium. To induce apoptosis in the positive controls, we added 10 μM staurosporine. The caspase activity was measured by luminescence, using a Veritas microplate luminometer (Turner Biosystems) with GloMax software at 60 minutes.

Western blot

Extracts from cultured immortalized lymphocytes were prepared with lysis buffer (PBS, 50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1× protease inhibitors, and 1× phosphatase inhibitors) or radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 10% sodium deoxycholate, 1% NP-40, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM polyvinylidene difluoride, and 1 mM NaF). Protein lysates in aliquots of 20 μg were separated by NuPAGE Novex 10% Bis-Tris gels (Invitrogen, CA, USA) and transferred to a polyvinylidene fluoride membrane. Primary antibodies used were the following: TCF7L2 (TCF4) (1:25 000; Abcam), chaperone BiP (1:1000) (Santa Cruz Biotechnology, Inc.), phosphor-eIF2α (1:1000; Cell Signaling Technology), CHOP (1:1000; Cell Signaling Technology), and the housekeeping actin (Sigma-Aldrich).

In silico analysis of splicing factor binding sites

Putative binding sites surrounding the *TCF7L2* alternatively spliced exons and intron boundaries were analyzed using 2 different software programs: SFmap (http://sfmap.technion.ac.il) and SpliceAid-F (http://srv00.ibbe.cnr.it/SpliceAidF/).

Statistical analyses

Statistical analyses were performed with GraphPad Prism (version 5; GraphPad Software, Inc.) and the Statistical Package for the Social Sciences (SPSS version 20; IBM Corporation). Data were analyzed with the Shapiro-Wilk test to assess the assumption of normality. For comparison of differences between the 4 groups of patients, ANOVA followed by a post hoc Tukey analysis was applied. In the case of deviation from a normal distribution, we used the Kruskal-Wallis and Dunn multiple comparison tests. For the cell transfection comparison, statistical significance was tested by the Mann-Whitney *U* test. A value of $P < .05$ was considered statistically significant. Data are presented as means ± SEM.

Results

Differential expression of the *TCF7L2* splice variants and protein, depending on the at-risk genotype

It has been demonstrated that *TCF7L2* presents a complex alternative splicing pattern (Supplemental Figure 1A), but to date, the splice variants that are preferentially expressed by the T2D risk-associated genotype in T2D patients and healthy carriers remain unknown. Therefore, we performed qRT-PCR to measure the expression of the *TCF7L2* splice variants in immortalized B lymphocytes from the 4 groups studied (Supplemental Figure 1B). We focused on exon 4, the 9 extra nucleotides at the end of exon 7, the 12 extra nucleotides at the beginning of exon 9, and the 3′ end variants, which comprise exons 12 to 17 (Figure 1A).

Alternative splicing of exon 4 has been extensively studied, because it has been thought to be associated with the at-risk *T/T* genotype (16). Our results showed that T2D patients carrying the at-risk *T/T* genotype (*T/T* T2D) had a statistically significant decrease in exon 4 exclusion ($P = .038$ vs T2D patients carrying the *C/C* genotype, hereafter *C/C* T2D). On the other hand, nondiabetic individuals carrying the same at-risk *T/T* genotype (hereafter *T/T* non-T2D) showed a statistically significant increase in exon 4 exclusion ($P = .046$ vs nondiabetic individual carrying the *C/C* genotype, hereafter *C/C* non-T2D) (Figure 1, B and C, respectively, and Supplemental Table 2). Furthermore, the differences were higher in the splicing of exon 4 between nondiabetic individuals and T2D patients carrying the at-risk *T/T* genotype ($P = .0001$).

The complex splicing pattern covering the *TCF7L2* gene involves the inclusion/exclusion of 9 extra nucleotides at the end of exon 7 and 12 extra nucleotides at the beginning of exon 9. On one hand, the expression pattern in T2D patients carrying the at-risk *T/T* genotype showed a statistical decrease in the exclusion of the extra nucleo-

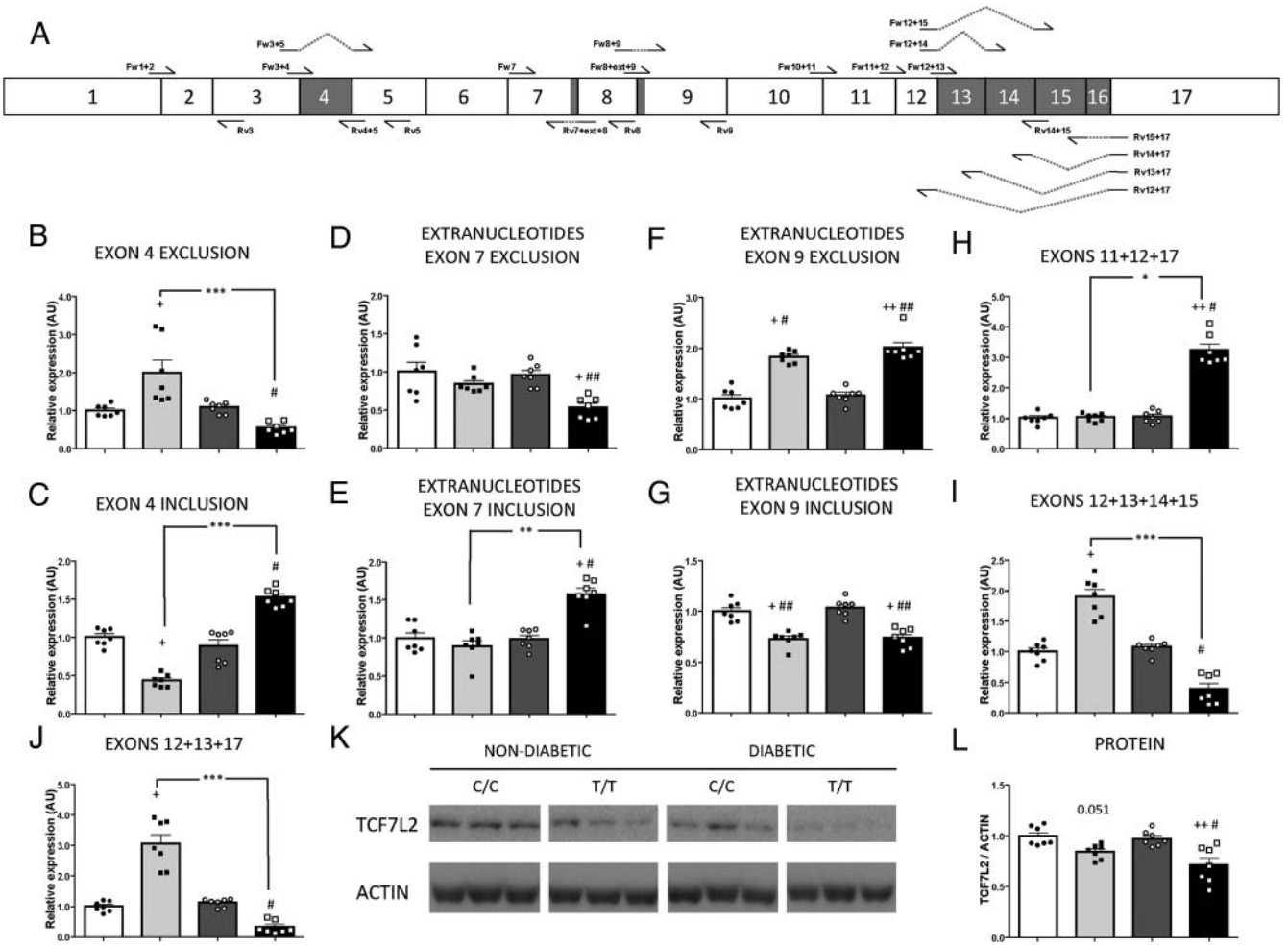

Figure 1. Differential expression of *TCF7L2* splice variants and protein levels. A, Structure of the *TCF7L2* gene. White bars indicate exons. Dark gray bars indicate alternatively spliced exons. Arrow show primer localization within the gene (exon-exon junction), and dashed lines indicate alternative splicing products. B–J, Graphical representations of the *TCF7L2* mRNA levels for the 4 groups studied: nondiabetic *C/C* (white bar with black circles), nondiabetic *T/T* (light-gray bar with black squares), diabetic *C/C* (dark-gray bar with white circles), and diabetic *T/T* (black bar with white squares), where the y-axis represents the relative expression of the analyzed splice variants of *TCF7L2* in arbitrary units (AU). Shown are exclusion (B) and inclusion (C) of exon 4, extra nucleotides at the end of exon 7 exclusion (D) and inclusion (E), 12 extra nucleotides at the beginning of exon 9 exclusion (F) and inclusion (G), and 3′ end splice variants: variant containing exons 11, 12, and 17 (H), variant containing all exons except 16 and 17 (I), and variant including exons 12, 13, and 17 (J). K, Representative Western blot for each group. L, Quantification of TCF7L2 protein, normalized by actin. Values are means ± SEM, representative of 2 independent experiments. +, $P < .05$ and ++, $P < .01$ vs *C/C* nondiabetic; #, $P < .05$ and ##, $P < .01$ vs *C/C* diabetic; *, $P < .05$, **, $P < .01$, and ***, $P < .001$ between nondiabetic and diabetic *T/T* carriers.

tides of exon 7 ($P = .003$ vs *C/C* T2D and $P = .012$ vs *C/C* non-T2D), whereas nondiabetic individuals carrying the same at-risk *T/T* genotype did not show statistical differences (Figure 1, D and E, respectively, and Supplemental Table 2). On the other hand, nondiabetic individuals and T2D patients carrying the at-risk *T/T* genotype showed a statistically significant increase in the exclusion of the extra nucleotides of exon 9 ($P = .021$ vs *C/C* non-T2D and $P = .003$ vs *C/C* T2D, respectively) (Figure 1, F and G, respectively, and Supplemental Table 2).

Regarding the splice variants covering the 3′ end of *TCF7L2*, the expression analysis showed a significant increase in the variant, including the junction between exons 12 and 17 in T2D patients carrying the at-risk *T/T* genotype ($P = .011$ vs *C/C* T2D, $P = .015$ vs *C/C* non-T2D and $P = .004$ vs *T/T* non-T2D) (Figure 1H and Supplemental Table 2). The expression of the splice variants including exons 12 to 15 or exons 12, 13, and 17 were significantly increased in nondiabetic individuals carrying the at-risk *T/T* genotype ($P = .042$ and $P = .035$ vs C/C non-T2D, respectively) but statistically decreased in T2D patients with the same at-risk *T/T* genotype ($P = .042$ and $P = .035$ vs C/C T2D, respectively) (Figure 1, I and J, respectively, and Supplemental Table 2). Nevertheless, we did not find any significant differences for the other splice variants studied, including exons 12, 14, and 17, exons 12, 15, and 17, or exons 12, 13, 14, and 17 (Supplemental Table 2).

In addition to the differential expression of the *TCF7L2* splice variants, we analyzed the protein levels, because it has been reported that TCF7L2 protein expression levels in the at-risk *T/T* carriers are lower than in *C/C* carriers. Our results show that protein expression was statistically decreased in T2D patients carrying the at-risk *T/T* genotype ($P = .008$ vs *C/C* T2D) and was also reduced in nondiabetic individuals carrying the at-risk *T/T* genotype ($P = .051$ vs *C/C* non-T2D) (Figure 1, K and L, and Supplemental Table 2).

TRA2B regulation of the *TCF7L2* exon 4 alternative splicing

Next, we aimed to investigate the cause for the differential *TCF7L2* splicing patterns, focusing our efforts on exon 4, since its splicing was significantly enhanced or repressed in both groups carrying the at-risk *T/T* genotype. Based on the in silico analysis of the binding sites of the splicing factor for *TCF7L2* exon 4 and the surrounding introns, we studied the heterogeneous nuclear ribonucleoprotein *hnRPA1* and the expression of the alternative splicing factors *SFRS7* and *TRA2B* (also known as *SFRS10*), taking into account the fact that *TRA2B* is an autoregulated splicing factor (31).

Interestingly, the expression analysis of the *hnRPA1* gene showed that nondiabetic individuals and T2D patients carrying the at-risk *T/T* genotype did not behave differently, because the gene is statistically significant decreased in both ($P = .002$ vs *C/C* non-T2D and $P = .028$ vs *C/C* T2D) (Figure 2A and Supplemental Table 3). On one hand, the alternative splicing factor *SFRS7* showed significant differences between nondiabetic individuals and T2D patients carrying the at-risk *T/T* genotype. *SFRS7* expression was increased in nondiabetic individuals ($P = .038$ vs *C/C* non-T2D), whereas it was reduced in T2D patients ($P = .031$ vs *C/C* T2D) (Figure 2B and Supplemental Table 3). On the other hand, the qRT-PCR results showed clear regulation of the alternative splicing factor *TRA2B* in T2D patients carrying the at-risk *T/T* genotype, with a significant increase in functional splice variant 1 (*TRA2B*-1, $P = .046$ vs *C/C* T2D) and a significant decrease in variant 4 (*TRA2B*-4, $P = .023$ vs *C/C* T2D), which is not translated into protein (Figure 2, C and D, respectively, and Supplemental Table 3). Therefore, the ratio *TRA2B*-1/*TRA2B*-4 (Figure 2E) was lower than 1 in nondiabetic individuals carrying the at-risk *T/T* genotype, which was the group that excludes more exon 4 in *TCF7L2*. However, in T2D patients carrying the at-

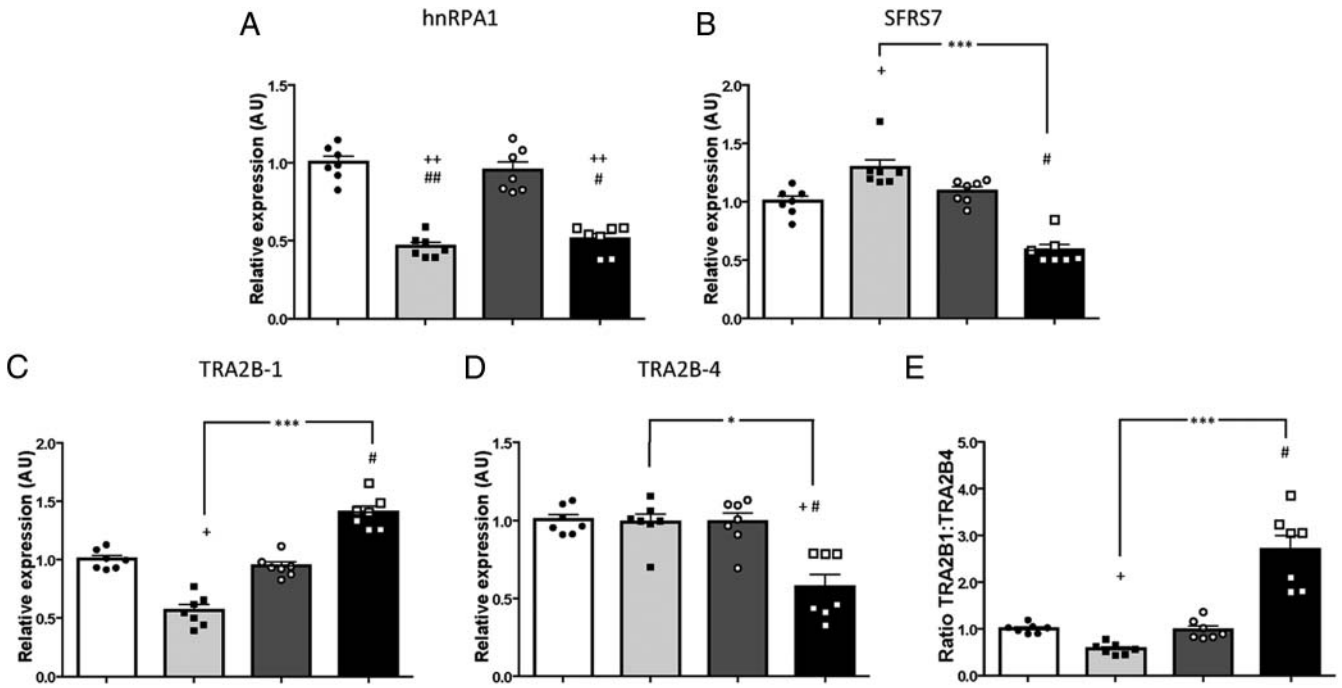

Figure 2. Down-regulation of alternative splicing factors in T2D risk-associated genotype carriers. Graphical representation of the relative expression of the genes encoding different splicing factors for the four studied groups: nondiabetic *C/C* (white bar with black circles), nondiabetic *T/T* (light-gray bar with black squares), diabetic *C/C* (dark-gray bar with white circles), and diabetic *T/T* (black bar with white squares), where the y-axis represents the relative expression of the analyzed genes in arbitrary units (AU). Regulation of the heterogeneous nuclear ribonucleoprotein hnRPA1 (A), the alternative splicing factors SFRS7 (B), and TRA2B variant 1 (C) and variant 4 (D). E, Graphical bar representation of the ratio of the functional variant TRA2B-1 and nonfunctional TRA2B-4. Values are means ± SEM, representative of 2 independent experiments. +, $P < .05$ and ++, $P < .01$ vs *C/C* nondiabetic; #, $P < .05$ and ##, $P < .01$ vs *C/C* diabetic; *, $P < .05$, **, $P < .01$, and ***, $P < .001$ between nondiabetic and diabetic *T/T* carriers.

risk *T/T* genotype, who had increased inclusion of *TCF7L2* exon 4, the ratio was higher than 1, meaning that in the second group there was a higher amount of *TRA2B*-1 mRNA, which was able to translate into functional protein.

Taking into account these interesting results and also the facts that the in silico analysis showed that TRA2B was the main transcription factor in this locus and that it has binding sites close to the SNP rs7903146, we decided to study whether *TRA2B* is an essential alternative splicing factor of *TCF7L2* exon 4. To address this hypothesis we silenced *TRA2B* in control nondiabetic individuals carrying the *C/C* genotype and studied whether this silencing was able to alter the splicing of *TCF7L2* exon 4. We found that the in vitro *TRA2B* silencing showed a significant reduction of both isoforms (*TRA2B*-1, $P = .0006$ and *TRA2B*-4, 68% $P = .0006$) (Figure 3, A and B, respectively, and Supplemental Table 4), triggering a significant decrease in *TC7L2* exon 4 exclusion ($P = .0041$) and a significant increase in exon 4 inclusion ($P = .0006$) (Figure 3, C and D, and Supplemental Table 4).

Given that these results shed some light on the possible mechanism but the main question remains still to be answered, we think that the regulation of this differential expression of the alternative splice variant could be due to an environmental risk factor, such as high levels of insulin (32, 33) or glucose. For this reason, we assessed the expression levels of *TRA2B* in an in vitro cell culture of control nondiabetic individuals carrying the *C/C* genotype with a high concentration of glucose (25 mM) or insulin (100 nM). Interestingly, the expression levels of *TRA2B* were significantly decreased under hyperglycemic and hyperinsulinemic conditions ($P = .015$ and $P = .018$ vs untreated *C/C* non-T2D, respectively) (Figure 3E and Supplemental Table 4).

ER stress pathways and translational regulation of TCF7L2

Proteins requiring posttranslational modifications are processed in the ER. Different cellular scenarios can lead to a dysfunction of the ER and ultimately to an imbalance between the protein-folding capacity and load, triggering ER stress. The first step is that the unfolded or misfolded proteins are tagged for degradation via the endoplasmic reticulum–associated degradation (ERAD) pathway. How-

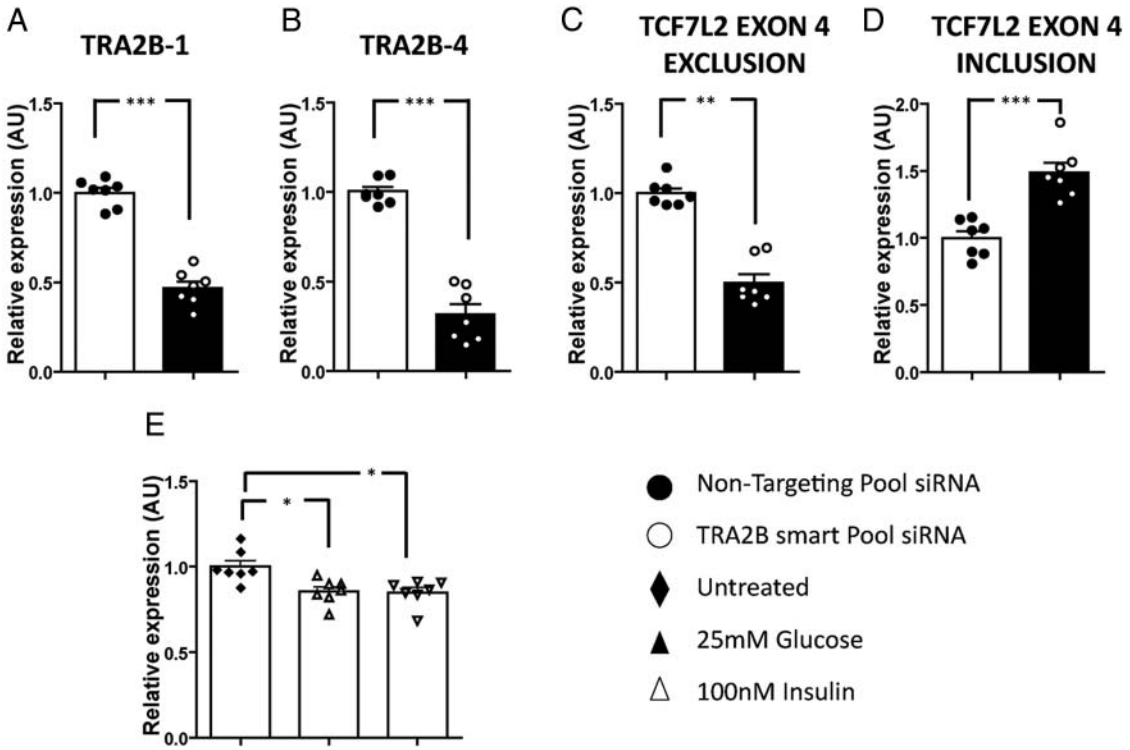

Figure 3. TRA2B silencing and *TCF7L2* exon 4 splicing. Graphical bar representation of the TRA2B splice variant 1 (A) and splice variant 4 (B) siRNA silencing and the corresponding expression levels of *TCF7L2* exon 4 exclusion (C) and inclusion (D). Nondiabetic *C/C* individuals treated with non-targeting pool siRNA (white bar with black circles) and nondiabetic *C/C* individuals treated with TRA2B smart pool siRNA (black bar with white circles), where the y-axis represents the relative expression in arbitrary units (AU). E, Graphical bar representation of *TRA2B* expression including control (white bar with black rhombus), hyperglycemic (25 mM; white bar with white triangle), and hyperinsulinemic (100 nM; white bar with inverted white triangle) conditions. Values are means ± SEM, representative of 2 independent experiments. *, $P < .05$, **, $P < .01$, and ***, $P < .001$.

ever, if incorrectly folded proteins continue to accumulate, the UPR is triggered and induces apoptosis.

As our earlier results showed, *TCF7L2* presented a genotype-dependent differential expression of the *TCF7L2* splicing pattern in lymphocytes. The characterization of proapoptotic splice variants and the reduced protein levels in T2D patients carrying the at-risk *T/T* genotype might suggest the involvement of ER stress, thus triggering apoptosis in these T2D patients. Hence, we studied the expression and protein levels of several genes encoding the principal proteins involved in the UPR.

First, we analyzed the expression of the gene encoding the chaperone BiP (also known as GRP78). During adaptation, the UPR tries to reestablish a folding homeostasis by inducing the expression of chaperones that enhance protein folding. We found a significant increase in nondiabetic individuals and T2D patients carrying the at-risk *T/T* genotype ($P = .035$ vs *C/C* non-T2D and $P = .002$ vs *C/C* T2D), suggesting that ER stress could be associated with the T2D risk–associated genotype (Figure 4A and Supplemental Table 5).

Second, we studied the 3 different pathways involved in the UPR. Initially we analyzed the pathway mediated by the transmembrane PERK. The results showed an up-regulation of *PERK*, *ATF4*, *CHOP*, and *BIM* genes (proapoptotic) acting at different levels of the apoptosis pathway in T2D patients carrying the at-risk *T/T* genotype ($P = .014$, $P = .011$, $P = .007$, and $P = .017$ vs *C/C* T2D, respectively) (Figure 4, B and E–G, respectively, and Supplemental Table 5). Accordingly, the antiapoptotic gene *BCL2* was down-regulated ($P = .012$ vs *C/C* T2D) (Figure 4 H and Supplemental Table 5).

We next analyzed the pathway that was mediated by IRE1. This pathway presents 2 different branches. The

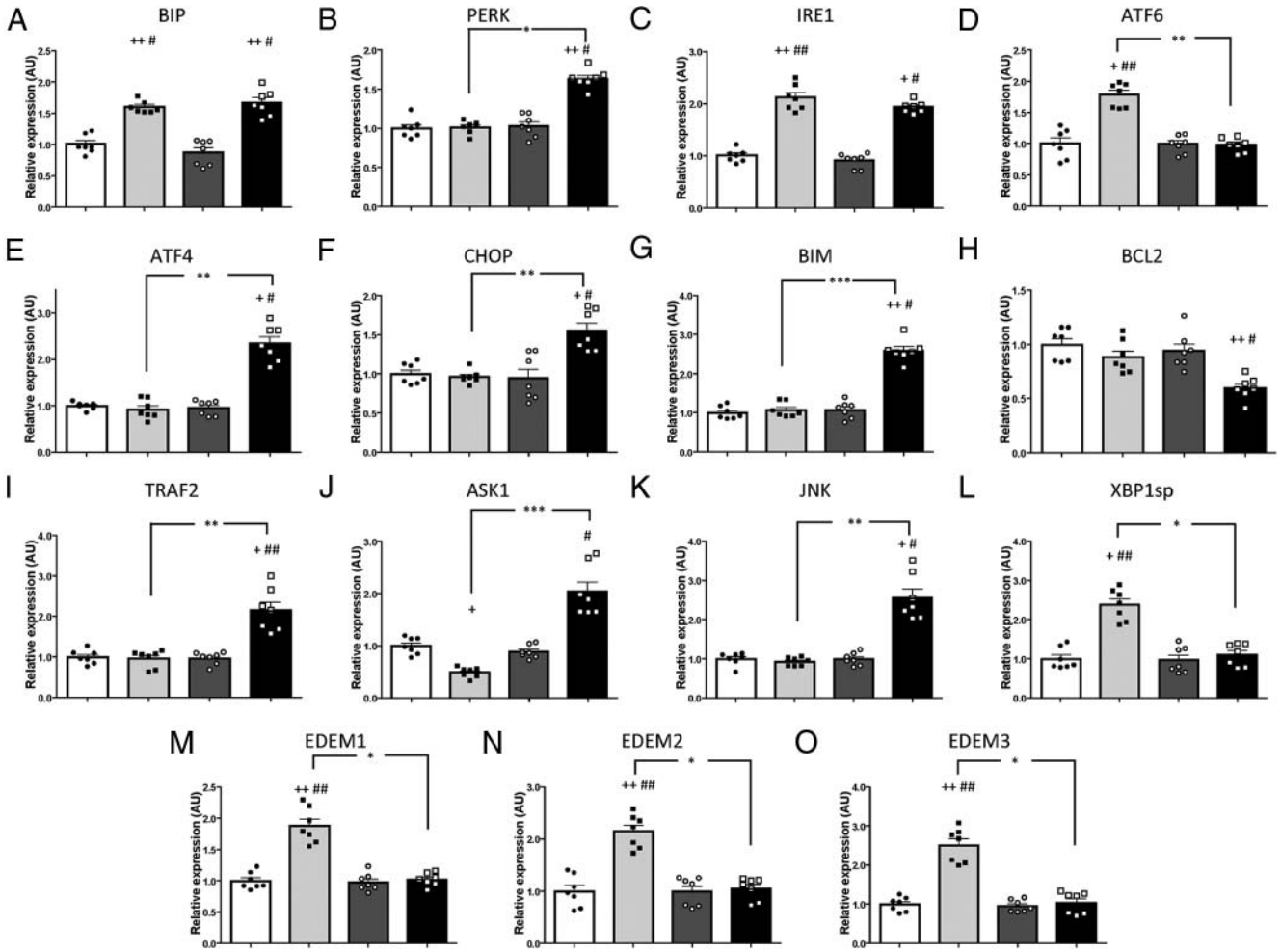

Figure 4. Expression of the ER stress genes. Panels show the graphical representation of the mRNA levels of the main genes involved in the ER stress pathways for the 4 groups studied: nondiabetic *C/C* (white bar with black circles), nondiabetic *T/T* (light-gray bar with black squares), diabetic *C/C* (dark-gray bar with white circles), and diabetic *T/T* (black bar with white squares), where the y-axis represents the relative expression of the analyzed genes in arbitrary units (AU). Shown are the chaperone BiP (A) and the 3 transmembrane receptors PERK (B), IRE1 (C), and ATF6 (D), its downstream genes ATF4 (E), CHOP (F), BIM (G), BCL2 (H), TRAF2 (I), ASK1 (J), JNK (K), XBP1sp (L), and EDEM 1 (M), EDEM2 (N), and EDEM3 (O). Values are means ± SEM, representative of 2 independent experiments. +, $P < .05$ and ++, $P < .01$ vs *C/C* nondiabetic; #, $P < .05$ and ##, $P < .01$ vs *C/C* diabetic; *, $P < .05$, **, $P < .01$, and ***, $P < .001$ between nondiabetic and diabetic *T/T* carriers.

first branch is mediated by the adaptor protein, TNF receptor–associated factor 2 (TRAF2), which activates the c-Jun NH_2-terminal kinase (JNK) through the apoptosis signal–regulating kinase (ASK1), leading to apoptosis. In this branch, all these proapoptotic genes were up-regulated in T2D patients carrying the at-risk *T/T* genotype (TRAF2, $P = .006$, ASK1, $P = .038$, and JNK, $P = .014$ vs *C/C* T2D) (Figure 4, C, I–K and Supplemental Table 5). The other branch is headed by X-box binding protein 1 (XBP1), which is spliced under ER stress, and the spliced isoform induces degradation via the ERAD pathway. Surprisingly, the spliced XBP1 was up-regulated in control nondiabetic individuals carrying the at-risk *T/T* genotype ($P = .012$ vs *C/C* non-T2D), whereas it remained unchanged in T2D patients carrying the at-risk *T/T* genotype. The spliced XBP1 results suggest that the nondiabetic individuals carrying the at-risk *T/T* genotype could be directed to the ERAD pathway instead of being directed to apoptosis (Figure 4L and Supplemental Table 5). To get a complete view of the ER stress pathway, we studied the ATF6 pathway, which showed an up-regulation of this gene in nondiabetic individuals carrying the at-risk *T/T* genotype ($P = .014$ vs *C/C* non-T2D) that finally leads to ERAD (Figure 4D and Supplemental Table 5). Because of the up-regulation of the genes involved in ERAD in control individuals carrying the at-risk *T/T* genotype, we analyzed the genes encoding 3 ER degradation enhancers (*EDEM1*, *EDEM2*, and *EDEM3*) and found that they were also up-regulated ($P = .007$, $P = .006$, and $P = .014$ vs *C/C* non-T2D, respectively) (Figure 4, M, N, and O, and Supplemental Table 5).

In addition to the differential mRNA expression of the genes involved in the ER stress pathway, we checked the protein levels of some of them. The protein levels of BiP ($P = .021$, *T/T* non-T2D vs *C/C* non-T2D and $P = .003$, *T/T* T2D vs *C/C* T2D), phospho-eIF2α (mediator of the PERK branch) ($P = .004$, *T/T* T2D vs *C/C* non-T2D), and CHOP ($P = .004$, *T/T* T2D vs *C/C* non-T2D) correlated with the previous mRNA expression results (Figure 5, A–D, and Supplemental Table 6).

Finally, apoptosis can also be measured by caspase activity and to confirm our previous results, we analyzed caspase activity using the Caspase-Glo 3/7 assay. Our results showed an increase of at least 1.7-fold in the caspase activity in T2D patients carrying the at-risk *T/T* genotype ($P = .010$ vs *C/C* T2D, $P = .003$ vs *C/C* non-

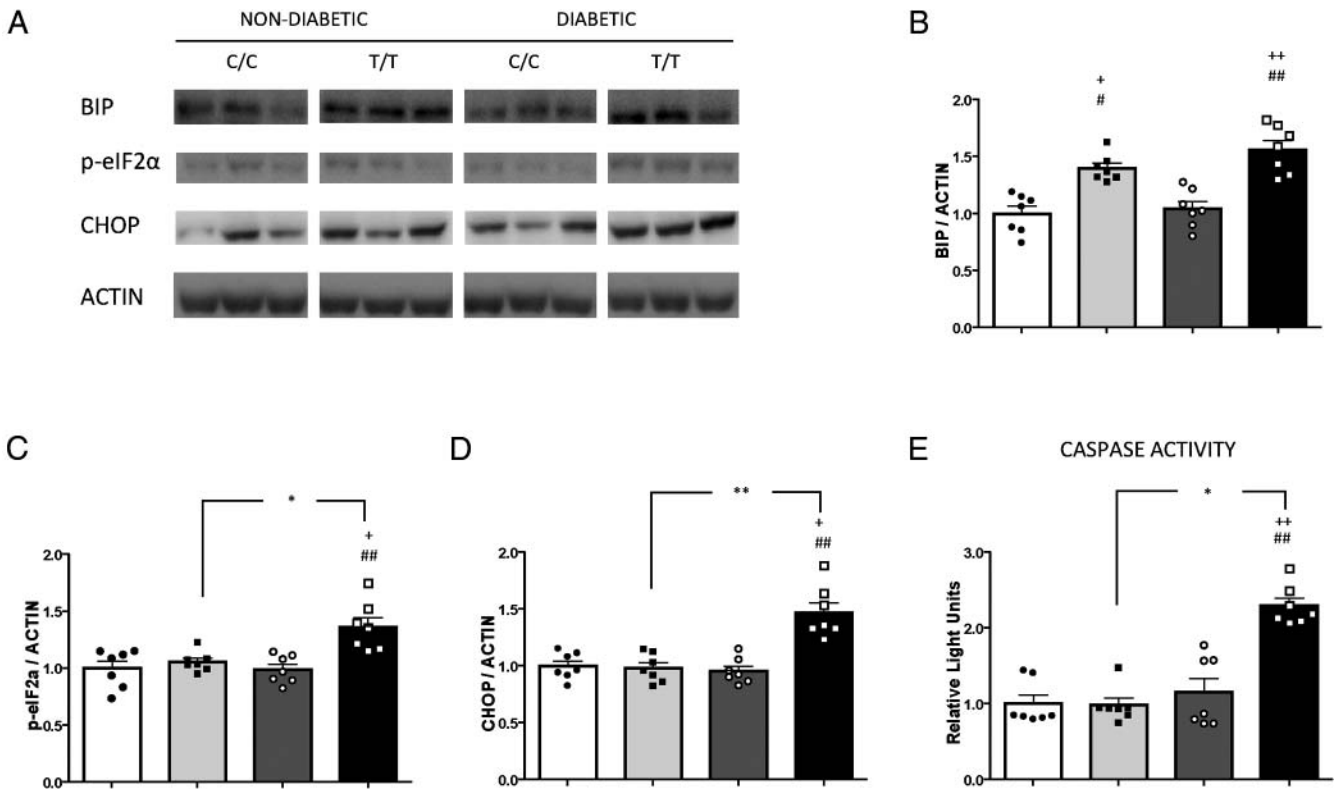

Figure 5. Protein levels of the ER stress response and caspase activity. A, Representative Western blots for the 4 groups studied: nondiabetic *C/C* (white bar with black circles), nondiabetic *T/T* (light-gray bar with black squares), diabetic *C/C* (dark-gray bar with white circles), and diabetic *T/T* (black bar with white squares), where the y-axis represents the relative quantification of the analyzed proteins. Quantification of protein levels of BIP (B), phospho (p)-eIF2α (C), and CHOP (D). E, Caspase 3/7 activity measured as relative light unit. Values are means ± SEM, representative of 2 independent experiments. +, $P < .05$, and ++, $P < .01$ vs *C/C* nondiabetic; #, $P < .05$ and ##, $P < .01$ vs *C/C* diabetic; *, $P < .05$ and **, $P < .01$ between nondiabetic and diabetic *T/T* carriers.

T2D, and $P = .021$ vs *T/T* non-T2D) (Figure 5E and Supplemental Table 6).

Discussion

SNPs in the *TCF7L2* gene (previously known as *TCF4*) have been shown to be strong genetic risk factors for T2D (3). The polymorphism with the strongest risk is the intronic SNP, rs7903146 (*T/T*); this SNP presents a T2D risk-associated genotype that is present in only 7%–10% of nondiabetic individuals but increases up to 14%–20% in T2D patients (3–5). During the last few years, efforts to understand the role of *TCF7L2* in the development of T2D have increased, and it has been shown that *TCF7L2* confers T2D risk mainly through impaired insulin secretion, perturbed incretin effect, and reduced β cell survival (34). In diabetes, *TCF7L2* mRNA is up-regulated in pancreatic islets, reducing glucose-stimulated insulin secretion, but TCF7L2 protein levels are down-regulated. One possibility for this controversial finding is that the increased mRNA expression represents multiple possible splice variants of *TCF7L2*, which encode many different isoforms with different effects on target cells.

Genetic variations may affect the levels of gene expression and splicing architecture of pre-mRNA transcripts, thus increasing the risk of development of a specific disease (3). In this regard, extensive work has been done to evaluate the splicing diversity of *TCF7L2* in a broad range of human and mouse cell lines and tissues, including not only pancreatic islets but also liver, gut, muscle, brain, and adipose tissues, among others (7–13, 15). Although several studies have revealed that *TCF7L2* presents a tissue-specific splicing pattern, most of these studies found no association between the splicing pattern and the T2D risk-associated *T/T* genotype. In this direction, Prokunina-Olsson et al (7) suggested an association of rs7903146 with the 3′ end splicing variants in pancreatic islets, pancreas, and colon (7), and Mondal et al (15) also found an association between the rs7903146 genotype and the 3′ end splicing variants in adipose tissue, but the statistical significance was lost after correction with multiple tests in both studies.

Taking into account the fact that opposite regulation of *TCF7L2* mRNA and protein levels exists in T2D (14, 27), we hypothesized that this opposite regulation behaves differently between nondiabetic individuals and T2D patients carrying the *C/C* or the at-risk *T/T* genotype. To address our hypothesis, we defined 4 study groups: nondiabetic individuals carrying (1) the nonrisk *C/C* genotype or (2) the at-risk *T/T* genotype and T2D patients carrying (3) the nonrisk *C/C* genotype or (4) the at-risk *T/T* genotype.

The ideal scenario to address our hypothesis would have been to study pancreatic β cells, which are the keystone cells in diabetes, because of their failure to compensate for high glucose levels in blood and initiation of the development of T2D. Considering the extremely low availability of human pancreas donors and the low frequency of this SNP in the population, we investigated splicing patterns in immortalized B lymphocytes. These cell lines have been extensively used as a source of biological material for functional and molecular studies and represent a potentially limitless source of genomic DNA, RNA, and protein. More precisely, in the scope of a study with an alternative splicing landscape (35–37), lymphocytes have been used as a reliable source. Indeed, it has been reported previously that pancreatic islets and blood have similar *TCF7L2* splicing patterns (7, 12), pointing to lymphocytes as a representative source for the study of alternative splicing. Osmark et al (12) reported that *TCF7L2* presents a tissue-specific expression pattern for exon 15, which is preferred in blood and islets, as well as for exon 4, which is retained principally in islets (~60%) but is also retained in blood (~35%). In their study, they did not observe a significant effect of the rs7903146 genotype on the splicing pattern, but interestingly they based this study on tissue samples from nondiabetic individuals exclusively, and, more remarkably, no individuals were homozygous for the risk T allele (12). Although immortalized B lymphocytes represent a convenient option to study a system in which one is not reliant on fresh tissue and experiments can be repeated consistently and this cell type has shown an alternative splicing pattern similar to that in pancreatic islets, we should be cautious when extrapolating results from lymphocytes to a plausible mechanism in pancreatic β cells. Lymphocytes cannot replace the β cell; however, lymphocytes could mirror some of the pathways and processes underlying the molecular mechanism, which explain how the *TCF7L2* genetic variation leads to altered biological functions in pancreatic β cells.

Our results showed increased inclusion of exon 4 in T2D patients carrying the at-risk *T/T* genotype, contrary to the increased exclusion of the same exon in nondiabetic individuals carrying the same at-risk genotype. Downstream we also found a different pattern for the extra nucleotides splicing into exon 7 and for the previously described 3′ end splicing variants. Interestingly, in 2011, Le Bacquer et al (13) described in human pancreatic β cells from nondiabetic pancreas donors, who did not carry the at-risk genotype, the presence of one proapoptotic *TCF7L2* splice variant (including exon 4 and skipping exons 13 to 16) and 2 other protective variants (that differed in the 3′ end splicing). We propose that in at-risk

T/T genotype carriers the T2D patients overexpressed the proapoptotic splice variant, whereas the control individuals overexpressed the protective splice variants. These results are a novel step in the understanding of the molecular mechanism that differs from the association between the SNP and T2D, because it points out the possibility of development of T2D in individuals with the at-risk *T/T* genotype but not in nondiabetic carriers.

Once we identified differential expression of *TCF7L2* splice variants between nondiabetic individuals and T2D patients carrying the at-risk *T/T* genotype and because several previous studies have been focused on the *TCF7L2* 3′ end splicing pattern, but no clear correlation between the splicing and the rs7903146 has been found, we focused our research on understanding the alternative splicing of exon 4. The in silico analysis of exon 4 and the intronic sequence surrounding rs7903146 showed an enrichment of several putative binding sites corresponding to a few splicing factors. Thus, we determined by qRT-PCR the expression levels of the alternative splicing factors *TRA2B* and *SFRS7* and the heterogeneous nuclear ribonucleoprotein *hnRPA1*, which had been shown previously by in silico analysis to bind to exon 4 and its surrounding intronic sequence. TRA2B is an SR-like protein that tightly autoregulates its own concentration by influencing the alternative splicing of its pre-mRNA (31). The proper concentration of TRA2B is important, since several pathological states are associated with changes in this concentration (32). In brief, the functional splice variant 1 (skips exon 2) encodes the protein TRA2B-1, which regulates the inclusion of its own exon 2. This inclusion of exon 2 generates mRNA (the nonfunctional splice variant 4 TRA2B-4) that is not translated into protein, so the proportion of both variants is important to keep the proper concentration of the TRA2B-1 protein.

We found differences in expression levels between non-T2D individuals and T2D patients carrying the at-risk *T/T* genotype. Nondiabetic individuals showed normal expression levels of TRA2B-4 and down-regulation of TRA2B-1, but, surprisingly, in T2D patients the results indicated a clear autoregulation of these splice variants. We found significant up-regulation in the expression of TRA2B-1 and significant down-regulation of TRA2B-4, keeping the ratio between the functional and nonfunctional isoforms (TRA2B1/TRA2B4) greater than 1 in T2D patients and lower than 1 in nondiabetic control individuals. When TRA2B was silenced in vitro in nondiabetic individuals carrying the *C/C* genotype, the exclusion of *TCF7L2* exon 4 was decreased, whereas the inclusion of exon 4 was increased. All of this information suggests that TRA2B plays an essential role, at least in human immortalized lymphocytes, in the splicing of *TCF7L2* exon 4.

Thus, we should consider the possibility that the natural development of T2D with increasing levels of glucose and insulin could be responsible for the changes in expression of the alternative splicing factors (32). Hence, different splice variants between nondiabetic controls and T2D patients are generated, as we have noted, finding a statistical reduction in TRA2B expression under hyperglycemic or hyperinsulinemic conditions. We should also take into account the fact that TRA2B is a splicing factor with complex autoregulation, and, as we proposed, the ratio between the functional and nonfunctional variants could be the keystone for the splicing of *TCF7L2* exon 4.

Furthermore, it is noteworthy that prior exposure to a poor metabolic environment could have generated a detrimental "metabolic memory", as has been described previously (38–43), including lymphocytes (44). This phenomenon has been replicated in animals and in several cellular models, indicating that expression patterns allow a quick response to changing environmental stimuli but at the same time confer to the cell the ability to "memorize" these encounters, deriving self-perpetuating modifications of gene expression patterns (45). Finally, we should keep in mind not only that the spliceosome machinery recognizes one specific exon along the *TCF7L2* gene but also that the spliceosome is built up of different constitutive and alternative splicing factors and TRA2B is just one of them.

Once we described the alternative splicing of *TCF7L2* exon 4, we focused our research on understanding the mechanisms underlying the posttranslational regulation of TCF7L2, in which protein levels are reduced in the at-risk genotype carriers. We considered that when these different splice variants are translated into proteins which are finally processed and folded in the ER, the ER faces some problems in proper folding of this variety of proteins. This process could trigger apoptosis through the UPR. Thus, we studied several genes implicated in the ER stress pathway and found that 2 different branches were overexpressed in the at-risk *T/T* genotype carriers, depending on whether they have developed T2D or not. On one hand, in nondiabetic individuals, the expression of *ATF6* and *IRE1*, through *XBP1* branches, was increased flowing into ERAD, including up-regulation of *EDEM1*, *EDEM2,* and *EDEM3*. On the other hand, in T2D patients the expression of *PERK* and *IRE1*, through *TRAF2* branches, was augmented. However, in this case, the up-regulation of the downstream genes, except for the anti-apoptotic *BCL2*, was associated with an increase in apoptosis, fitting with our previous results showing an

overexpression of the proapoptotic splice variant in T2D risk-associated genotype carriers (Supplemental Figure 2).

Based on the in silico and experimental data results, we speculate that the at-risk *T/T* genotype from the SNP rs7903146 in *TCF7L2* intron 4 could interact with an ISE recognized by the SR protein TRA2B, modulating the alternative splicing of *TCF7L2* exon 4. In addition, TRA2B not only recognizes this ISE but also recognizes ESEs in exon 4, which also promotes the inclusion of *TCF7L2* exon 4 (Supplemental Figure 3). In this hypothetical scenario, SFRS7 and hnRPA1 could also be involved in orchestrating the alternative splicing of *TCF7L2* exon 4. Taking this information into account, individuals carrying the *C/C* genotype (nondiabetic individuals and T2D patients) would not present interactions between TRA2B and the ISE. Therefore, regarding exon 4, they could be compensating between the enhanced exon 4 inclusion from TRA2B and the inhibited inclusion from SFRS7, with a final readout of a compensatory response between inclusion and exclusion (Supplemental Figure 3A). Alternatively, nondiabetic individuals carrying the at-risk *T/T* genotype could present interactions between TRA2B and the ISE. Therefore, regarding exon 4, SFRS7 presents higher expression than TRA2B, although a lower level of hnRPA1 would help to increase the exclusion of exon 4 (Supplemental Figure 3B). Finally, T2D patients carrying the at-risk *T/T* genotype could present a stronger interaction between TRA2B and the ISE (reinforced under diabetic conditions), and regarding exon 4, SFRS7 and hnRPA1 could present lower expression levels than TRA2B, which also binds tightly to the ISE, finally increasing the inclusion of exon 4 (Supplemental Figure 3C).

In summary, this study shows, for the first time, differences in the expression of the different *TCF7L2* splice variants in at-risk *T/T* genotype carriers that distinguish between nondiabetic and T2D patients. These differences are marked at the exon 4 splicing, indicating increased exclusion in T2D patients and increased inclusion in nondiabetic individuals carrying the at-risk *T/T* genotype. These splicing differences could be explained by the autoregulation of the alternative splicing factor TRA2B, which at the same time is regulated by glucose and insulin. The distribution of the splice variants is associated with previously described protective and proapoptotic *TCF7L2* splice variants. This association and the reduced protein levels found in at-risk *T/T* genotype carriers directed our study on ER stress. Our results suggest that the *TCF7L2* splice variants activate specific ER stress pathways in nondiabetic individuals and T2D patients carrying the at-risk *T/T* genotype, driving the increased apoptosis in T2D patients.

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M.P.-J., N.N., E.F.-R., and R.G. designed the project. M.P.-J., N.N., and E.F.-R. performed the research and analyzed the data. M.P.-J., E.F.-R. and R.G. wrote the manuscript. All authors have approved the final version of the manuscript.

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