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# RNA Sequencing Identifies Dysregulation of the Human Pancreatic Islet Transcriptome by the Saturated Fatty Acid Palmitate

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**Pancreatic β-cell dysfunction and death are central in the pathogenesis of type 2 diabetes (T2D). Saturated fatty acids cause β-cell failure and contribute to diabetes development in genetically predisposed individuals. Here we used RNA sequencing to map transcripts expressed in five palmitate-treated human islet preparations, observing 1,325 modified genes. Palmitate induced fatty acid metabolism and endoplasmic reticulum (ER) stress. Functional studies identified novel mediators of adaptive ER stress signaling. Palmitate modified genes regulating ubiquitin and proteasome function, autophagy, and apoptosis. Inhibition of autophagic flux and lysosome function contributed to lipotoxicity. Palmitate inhibited transcription factors controlling β-cell phenotype, including PAX4 and GATA6. Fifty-nine T2D candidate genes were expressed in human islets, and 11 were modified by palmitate. Palmitate modified expression of 17 splicing factors and shifted alternative splicing of 3,525 transcripts. Ingenuity Pathway Analysis of modified transcripts and genes confirmed that top changed functions related to cell death. Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of transcription factor binding sites in palmitate-modified transcripts revealed a role for PAX4, GATA, and the ER stress response regulators XBP1 and ATF6. This human islet transcriptome study identified novel mechanisms of palmitate-induced β-cell dysfunction and death. The data point to cross talk between metabolic stress and candidate genes at the β-cell level.**

Pancreatic β-cells are long-lived cells (1) that face protracted metabolic challenges in insulin-resistant individuals (2). This includes the chronic exposure to saturated free fatty acids (FFAs), present in a high-fat Western diet and released from the adipose tissue in obesity (3). High levels of saturated FFAs are predictive of the future development of type 2 diabetes (T2D) (3). High-fat feeding impairs the ability of β-cells to compensate for insulin resistance (4,5). Prolonged exposure to FFAs impairs insulin secretion in vivo and in vitro (6,7) and induces β-cell death (8) in a phenomenon called lipotoxicity.

Palmitate is the most common saturated FFA in man and has been used in in vitro studies to examine the mechanisms of lipotoxicity. Palmitate functionally impairs β-cells by inhibiting insulin transcription (9), inducing mitochondrial uncoupling (10), and inhibiting exocytosis by disrupting the coupling between $Ca^{2+}$ channels and insulin granules (11). The production of reactive oxygen species

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(10) and ceramides (12) has also been implicated in palmitate-induced β-cell dysfunction and death. We, and others, have previously shown that FFAs induce endoplasmic reticulum (ER) stress in β-cells (13–16). The ER plays a central role in the synthesis and folding of secretory proteins. In β-cells, insulin represents up to 50% of the protein synthesized (17). ER stress, defined as an imbalance between protein folding demand and ER capacity, leads to accumulation of misfolded proteins. ER stress is sensed by the ER stress transducers PERK, IRE1, and ATF6 that activate the unfolded protein response (UPR). The UPR attenuates protein translation to relieve the load on the ER and induces ER chaperones, ER-associated degradation, and ER expansion. UPR is an adaptive response but triggers apoptosis when prolonged or exaggerated. Saturated FFAs elicit marked PERK activity, and the resulting eIF2α phosphorylation contributes to β-cell death (13,18) by the mitochondrial apoptosis pathway (19).

Unbiased approaches to examine the β-cell response to palmitate include microarray studies of clonal INS-1 and MIN6 cells. These studies showed induction of genes involved in FFA β-oxidation, FFA desaturation, steroid biosynthesis, cell cycle, chemokines, and acute-phase response genes, and inhibition of genes involved in glycolysis and aminoacyl tRNA biosynthesis (20–23). Our time course microarrays of palmitate-treated INS-1E cells led to the identification of the proapoptotic Bcl-2 proteins that mediate lipotoxic β-cell death (19). Oleate-treated human islet arrays showed transcriptional induction of FFA oxidation, inflammatory genes, and antioxidant enzymes (24).

One of the intrinsic limitations of microarrays is that transcript detection is limited to transcripts for which probes are present on the arrays. RNA-sequencing (RNA-seq) has become the gold standard for transcriptomic studies, allowing detection of low-expressed genes, alternative splice variants, and novel transcripts (25). Our group has used RNA-seq to map the transcriptome of human islets (26), and this has been recently replicated in human islets and purified human β-cells (27); the latter study showed that islets are a good proxy for β-cell transcript expression.

Environmental challenges interact with the genetic background of individuals to generate disease. Recent genome-wide association studies (GWAS) have linked a number of genetic variants to susceptibility to T2D (28). Many of these variants seem to be related to pancreatic β-cell function, but there is little information on the expression and function of these genes in human β-cells faced with prolonged metabolic stress. The known candidate genes for T2D explain less than 10% of the heritability of the disease (28). Epigenetic alterations, such as changes in DNA methylation, have been described in pancreatic islets from T2D patients (29) and may explain part of the missing heritability. Whether the expression of these epigenetic T2D candidate genes is modified by metabolic stress has never been investigated.

To better understand the global responses of human islets exposed to metabolic stress, we have used RNA-seq to identify all transcripts, including splice variants, expressed in human islets of Langerhans after a 48-h exposure to the saturated FFA palmitate. This in vitro model of lipotoxicity arguably induces more rapid and harmful effects than those that may occur in vivo. Nonetheless, this analysis provides a snapshot of the cellular responses under conditions that may prevail in T2D. Key findings were validated and followed up by functional studies in independent human islet samples and clonal or primary rat β-cells. We also examined whether putative candidate genes for T2D are expressed in human islets and modified by palmitate.

## RESEARCH DESIGN AND METHODS

### Human and Rat Islet Isolation and Cell Culture

Human islet collection and handling were approved by the local ethical committee in Pisa, Italy. Human pancreatic islets were isolated in Pisa using collagenase digestion and density gradient purification from beating-heart organ donors with no medical history of diabetes or metabolic disorders (30). The donor characteristics are provided in Supplementary Table 1. The first five preparations (2 women and 3 men, donor age 55 ± 9 years, BMI 24.8 ± 0.7 kg/m$^2$) were used for RNA-seq, and the other preparations (15 women and 16 men, donor age 63 ± 3 years, BMI 25.0 ± 0.5 kg/m$^2$) were used for confirmation and functional studies. The islets were cultured in M199 culture medium containing 5.5 mmol/L glucose in Pisa and were shipped to Brussels within 1–5 days of isolation. In Brussels, the human islets were cultured in Ham's F-10 medium containing 6.1 mmol/L glucose, 10% heat-inactivated FBS, 2 mmol/L GlutaMAX, 50 μmol/L 3-isobutyl-1-methylxanthine, 1% charcoal-absorbed BSA, 50 units/mL penicillin, and 50 μg/mL streptomycin.

Islet β-cell purity was evaluated in dispersed islet cells by insulin immunocytochemistry and averaged 52 ± 3% (26). The islets were exposed or not to 0.5 mmol/L palmitate in the same medium without FBS for 2 days (8,13,31). The serum-free culture conditions have previously been validated (32). Human islet viability, assessed after Hoechst 33342 and propidium iodide staining, was similar in serum-free or 10% FBS-containing medium (respectively 93 ± 1% vs. 95 ± 1% viable cells after 72 h, $n$ = 8; $P$ = 0.3).

Rat insulin-producing INS-1E cells (provided by C. Wollheim, University of Geneva, Geneva, Switzerland) (33) were cultured in RPMI 1640 medium supplemented with 5% FBS, 10 mmol/L HEPES, 1 mmol/L Na-pyruvate, and 50 μmol/L 2-mercaptoethanol (34). The INS-1E cells were exposed to palmitate, as described (13).

Rats were used according to the Belgian Regulations for Animal Care with approval of the Ethical Committee for Animal Experiments of the Université Libre de Bruxelles, Brussels, Belgium. Islets were isolated from adult male

Wistar rats (Charles River Laboratories, Brussels, Belgium), and primary β-cells were fluorescence-activated cell sorter–purified (FACSAria; BD Bioscience, San Jose, CA) and cultured as described (13).

### RNA-seq and Data Analysis

Five human islet preparations were sequenced and data analyzed as previously described in detail (26). In brief, polyA-selected mRNA was purified from total RNA isolated with the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). mRNAs were reverse-transcribed to cDNA, paired-end repaired, 3′-monoadenylated, and adaptor-ligated. cDNA products (200 bp) were amplified and libraries submitted to quality control with the Agilent 2100 Bioanalyzer (Agilent Technologies, Wokingham, U.K.). The RNA integrity number (RIN) values for all samples were >7.5. cDNA was sequenced on one sequencing lane of an Illumina Genome Analyzer II system (Illumina). The raw data will be deposited in Gene Expression Omnibus (GEO), submission number GSE53949.

Paired-end reads were mapped to the human genome (version GRCh37/hg19) using gem-mapper from the Genomic Multitool (GEM) suite (http://gemlibrary.sourceforge.net). Mapped reads were used to quantify transcripts from the RefSeq reference database (35), using the Flux Capacitor (http://flux.sammeth.net) (36). Genes and transcripts were assigned a relative coverage rate as measured in RPKM units ("reads per kilobase of exon model per million mapped reads") (37). Lists of differentially expressed genes and transcripts were generated from the Flux Capacitor output using scripts in Perl or R. Palmitate-modified genes were defined by taking the $\log_2$ of the proportion between the sum of the RPKM for all gene transcripts under palmitate condition and control condition. A Fisher exact test (number of reads mapped to the gene and number of reads mapped to all other genes in palmitate vs. control) was Benjamini-Hochberg–corrected (taking for each gene the five samples as independent tests), and a difference in gene expression was considered significant if the $P$ value was <0.05. A gene was considered modified by palmitate if its expression changed significantly in one direction in at least four of five islet preparations and no significant change in the opposite direction was observed. Differences in splice indices—the proportion between the RPKM for a transcript and the sum of the RPKM for all the transcripts from the same gene—were compared between the palmitate and control condition. Splicing analysis was done using GENCODE version 16 annotations data sets (38–40). The GENCODE annotation data set used contains 153,008 transcripts, corresponding to 25,492 protein coding genes and long intergenic noncoding RNAs. Changes in splicing were statistically tested as above; that is, by Benjamini-Hochberg–corrected Fisher exact test-defined $P$ value <0.05 in at least four of five islet samples and no sample pair exhibiting a significant change in the opposite direction.

### Human Islet and Rat β-Cell RNA Extraction and RT-PCR

Human islets (Supplementary Table 1), INS-1E, and primary rat β-cells were used for validation and mechanistic experiments. PolyA mRNA was isolated using the Dynabeads mRNA DIRECT kit (Invitrogen, Paisley, U.K.) and reverse-transcribed as previously described (34). Quantitative (q)RT-PCR was done using the iQ SYBR Green Supermix (Bio-Rad, Nazareth Eke, Belgium) on a LightCycler (Roche Diagnostics, Mannheim, Germany) or iCycler MyiQ Single Color (Bio-Rad) instrument (41,42). Data were expressed as number of copies using the standard curve method and corrected for the housekeeping gene β-actin or GAPDH. Primers used for qRT- and RT-PCR are listed in Supplementary Table 2.

### RNA Interference

Human and rat β-cells were transfected with 30 nmol/L small interfering RNA (siRNA) and lipofectamine RNAiMAX (Invitrogen) diluted in Opti-MEM I (Invitrogen) as described (43), resulting in a transfection efficiency of >90% (43,44). After overnight transfection, the cells were cultured for 48 h before further use. The siRNAs are listed in Supplementary Table 3.

### Western Blot

Western blots were performed using equal amounts of whole-cell extract protein as described (18). Briefly, cell lysates were run on SDS-PAGE, washed in transfer buffer, and proteins were transferred to a nitrocellulose membrane. The primary antibodies were anti–β-actin (1:2,000), GATA6, and LC3B (both 1:1,000; Cell Signaling Technology, Beverly, MA); LONP1 (Proteintech Group), and anti–α-tubulin (1:10,000; Sigma-Aldrich). Horseradish peroxidase-labeled donkey anti-rabbit or donkey anti-mouse (1:10,000, Jackson ImmunoResearch, West Grove, PA) antibodies were used as secondary antibodies. Protein signal was visualized using chemiluminescence SuperSignal (Pierce) and quantified using Scion Image (Scion Corp., Frederick, MD).

### Assessment of Apoptosis

The percentage of apoptotic cells was determined in at least 500 cells per condition by staining with the DNA-binding dyes propidium iodide and Hoechst 33342 (Sigma-Aldrich), as previously described (18).

### Electron Microscopy

Electron microscopy studies were performed on isolated human islets as previously described (30).

### Statistical Analyses

The statistical analyses of the RNA-seq data are described in RNA-SEQ AND DATA ANALYSIS. Data for confirmation and functional studies are shown as means ± SEM. Comparisons were performed by paired two-tailed Student $t$ test or ratio $t$ test. A $P$ value ≤ 0.05 was considered statistically significant.

## RESULTS

### Sequencing of Palmitate-Treated Human Islets and Analysis of Transcripts

Five human islet preparations, exposed or not to palmitate for 48 h, were RNA-seq. The characteristics of the organ donors and islet preparations are presented in Supplementary Table 1. The percentage of cell death in palmitate-treated human islets was 12 ± 2%, compared with 6 ± 2% in the control condition (48 h, $P < 0.05$). The reads were mapped to the human genome (version GRCh37/hg19) using GEM software, mapping on average 85% of raw reads. Transcript expression and splicing was evaluated using Flux Capacitor software. As a reference transcript annotation, we used the 42,012 annotated human mRNA and noncoding RNA sequences from RefSeq.

Of the 18,463 genes detected by the RNA-seq, 1,325 (7%) were significantly modified by a 48-h exposure to palmitate, with 428 being upregulated and 897 being downregulated. Compared with our previous RNA-seq analysis of cytokine-exposed human islets (26), there was limited overlap between the two stress conditions (Supplementary Fig. 1*A*–*C*). Of the genes upregulated by cytokines, 10% were also induced by palmitate, and of the cytokine-inhibited genes, 19% were palmitate-regulated, showing that palmitate induced specific transcript expression changes.

The genes detected as modified by palmitate by RNA-seq were compared with microarray data of human islets from T2D donors and/or donors with $HbA_{1c} \geq 6\%$ (45). Of the genes differentially expressed in T2D and hyperglycemia, 7–16% were modified by palmitate in nondiabetic islets. In 82% of these genes, the change in expression occurred in the same direction for palmitate exposure and T2D (Supplementary Fig. 1*D*).

For internal validation, expression data were confirmed for seven genes by qRT-PCR in the same islet samples used for RNA-seq. The gene expression data were essentially superimposable (Supplementary Fig. 2). Additional validation was done by comparing RNA-seq data with qRT-PCR in independent human islet samples for 30 genes, showing a correlation coefficient of 0.63 (Supplementary Fig. 2).

### Expression of Candidate Genes for T2D in Human Islets

We examined whether known T2D candidate genes (28) are expressed (median RPKM >1) in human islets. We defined a set of 69 genes using the convention typically used in naming GWAS loci (i.e., in the absence of a strong biological candidate to choose the nearest gene to the peak GWAS signal). It is likely that this gene set is enriched for transcripts that mediate the GWAS locus effects. We did not exclude genes from loci that have been shown to act through nonislet mechanisms. Of the 69 candidate genes, 59 (86%) were present in human islets (Fig. 1*A*). This was a significantly higher proportion than that of candidate genes associated with ulcerative colitis (46) or body height (47), or a random set of 60 genes (Fig. 1*B*). The T2D genes were also more abundantly expressed with higher RPKM. Similarly, a large proportion of genes previously identified to harbor differential DNA methylation in T2D islets (29) were well expressed in human islets (Supplementary Fig. 3), with median expression of 8 RPKM compared with 6 RPKM for all detected genes.

### Analysis of Palmitate-Modified Genes

We next analyzed the human islet genes that were modified by palmitate (complete list accessible at http://lmedex.ulb.ac.be/data.php, with password provided on request). These genes were analyzed using Ingenuity Pathway Analysis (IPA; Supplementary Fig. 4) and Database for Annotation, Visualization and Integrated Discovery (DAVID) software, and they were manually curated (Supplementary Table 4 and Fig. 2). IPA showed that upregulated genes belong to the functions "Cell Death," "Cellular Movement" (mainly chemokines), "Cellular Development," "Gene Expression," and "Lipid Metabolism" (Supplementary Fig. 4*A*). Downregulated genes fell into the functional categories "Cellular Movement," "Cell Morphology," "Lipid Metabolism," "Molecular Transport," and "Small Molecule Biochemistry" (Supplementary Fig. 4*B*). The manual annotation was performed taking a pancreatic β-cell perspective. It showed induction of genes involved in lipid metabolism, including the transcription factor SREBP2, and early response genes that are part of an adaptive response (Fig. 2). Palmitate inhibited expression of key β-cell transcription factors, including PDX1, PAX4, MAFA, and MAFB, hormones and receptors, genes involved in ATP production, and channels and transporters, thereby likely contributing to induce β-cell dysfunction. Upregulation of a large number of UPR genes and inhibition of protein degradation pathways are likely to further contribute to β-cell dysfunction and death. Growth and regeneration genes were inhibited. Inflammatory responses were extensively modified, with upregulation of cytokines and chemokines and inhibition of HLA (Supplementary Table 4 and Fig. 2).

### FFA Metabolism

Palmitate exposure induced gene expression of fatty acid metabolic pathways (Fig. 3*A*). It induced ACSL1 and ACSL3, involved in FFA activation to acyl-CoA moieties, CPT-1, which mediates mitochondrial FFA uptake, ACADVL, ECH1, and HADHA, three enzymes involved in mitochondrial FFA β-oxidation, and two FFA desaturases that introduce double bonds into saturated FFAs and, as such, are β-cell protective (21). Interestingly, palmitate inhibited expression of enzymes involved in the de novo synthesis of ceramide (including SERINC5, SPTSSB, and CERS2). In parallel, it inhibited genes involved in the lysosomal breakdown of ceramide and sphingolipids (Fig. 3*A*).

### Protein Synthesis/Processing and ER Stress

Palmitate induced the aminoacyl tRNA synthetases IARS, GARS, MARS, WARS, VARS, CARS, and SARS and the translation elongation factor EEF1A2, involved in delivery of aminoacyl tRNAs to the ribosome. Protein translation

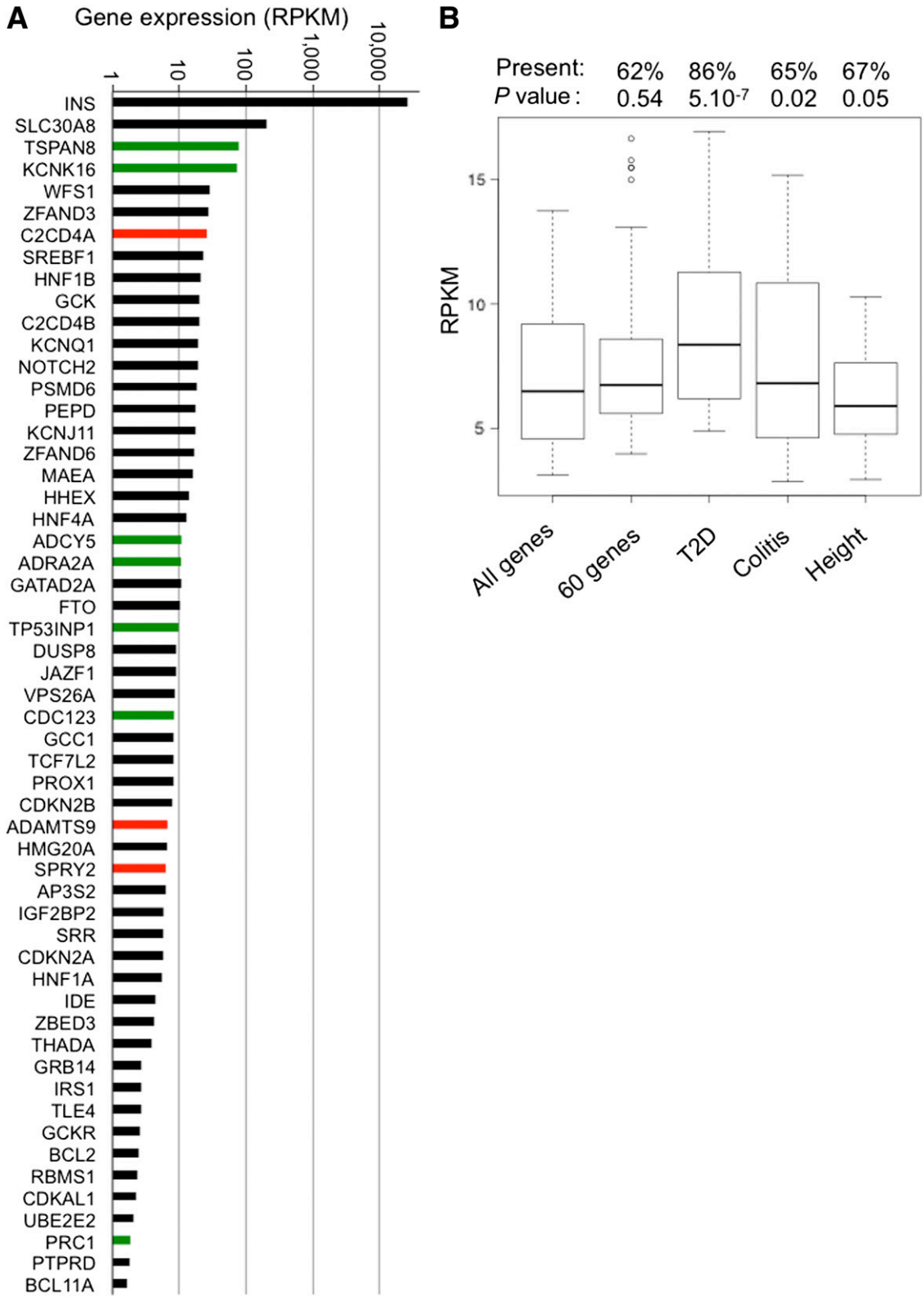


**Figure 1**—GWAS-based T2D candidate genes are well expressed in human islets. *A*: Expression levels of transcripts of T2D candidate genes with an expression of RPKM >1 (i.e., 80% of currently known T2D candidate genes). Red bars indicate significantly upregulated transcripts and green bars, downregulated transcripts. *B*: Box plot of median expression levels of all T2D candidate genes compared with all genes, a random set of 60 genes, or the candidate genes associated with ulcerative colitis or body height. The numbers above the figure show the percentage of genes considered present (RPKM >1) and the *P* value for the Fisher exact test of the selected gene set vs. all genes. The horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; the whiskers mark the highest and the lowest data point still within 1.5 interquartile range above the 75th percentile and 1.5 interquartile range below the 25th percentile, respectively; and the circles indicate outliers.

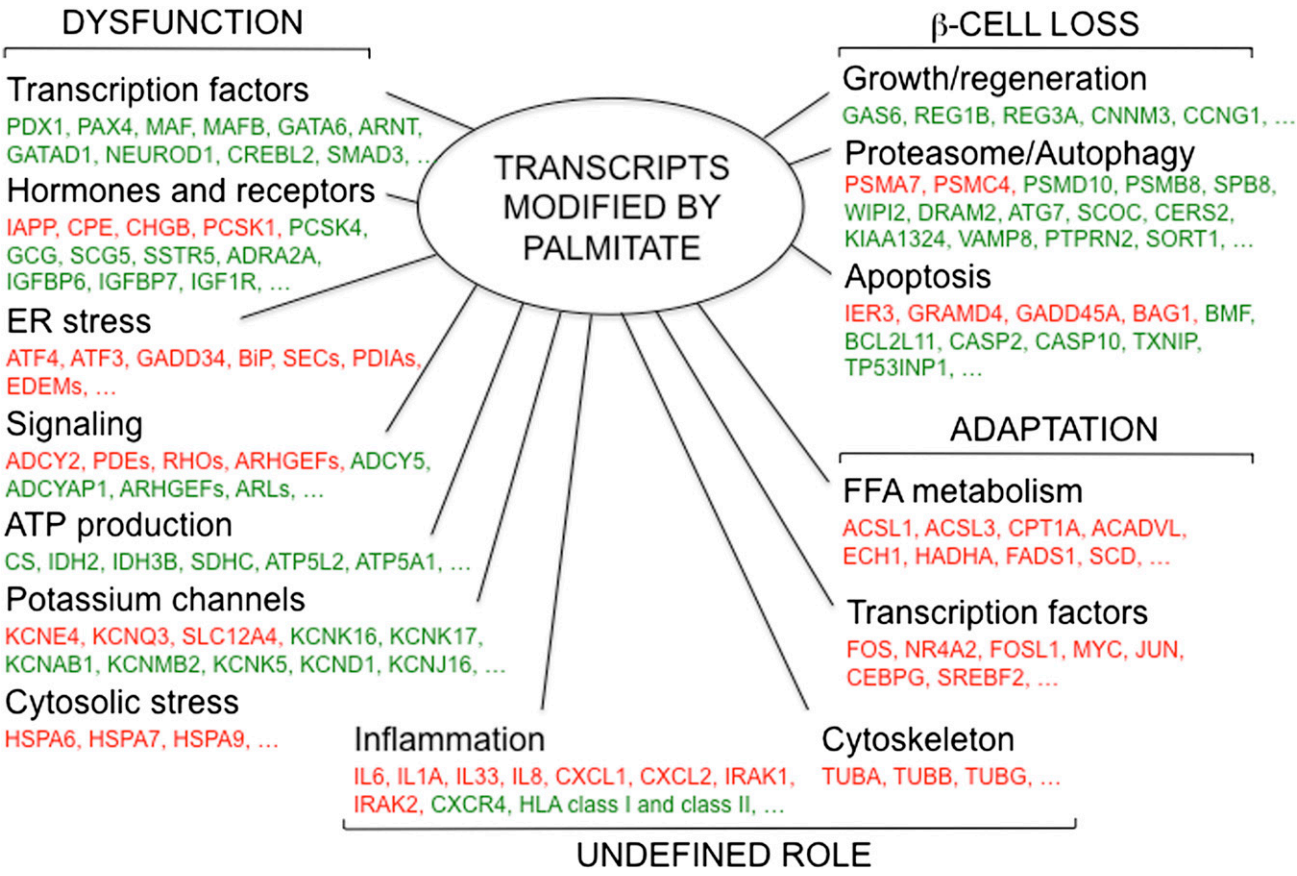


**Figure 2**—Overview of human islet transcripts modified by palmitate. Manual curation of RNA-seq–detected human islet transcripts modified by a 48-h exposure to palmitate into functional categories and β-cell outcomes. Upregulated genes are shown in red and downregulated genes in green. Expression changes leading to β-cell dysfunction include inhibition of key β-cell transcription factors, changes in hormones and receptors, the ER stress response, β-cell signal transduction, inhibition of ATP production, potassium channels, and a cytosolic stress response. Other transcript changes may contribute to β-cell loss, including inhibition of cell growth and regeneration factors, inhibition of autophagy, and changes in apoptosis-related genes. Adaptive responses are those related to FFA metabolism and early response transcription factors. The potential role and outcome of the induction of tubulin transcripts, the upregulation of innate immunity, and the downregulation of HLA are undefined.

initiation factors were modulated, with induction of EIF4A1 and inhibition of EIF4A2 expression, and inhibition of the translational repressor EIF4G2. Hormone processing was affected with an induction of CPE and PCSK1 and inhibition of PCSK4 and SCG5 (Fig. 3*B*).

The RNA-seq data indicated transcriptional activation of the three branches of the UPR, including PERK-dependent induction of ATF4, ATF3, TRIB3, and GADD34; ATF6-dependent induction of BiP and its cofactor DNAJB11; and IRE1-dependent induction of chaperones and protein disulfide isomerases (Fig. 3*B*). In line with this, DAVID analysis using UCSC_TFBS showed enrichment for potential binding sites for the transcription factors ATF6 and IRE1-dependent XBP1 (Fig. 4*A*).

Interestingly, palmitate induced expression of the ER stress transducers CREB3 and CREB3L3 (Fig. 3*B*), which may play roles similar to ATF6 in a tissue-specific way (48,49). Because their role in β-cells is unknown, we studied these ER stress transducers further. CREB3 was well expressed in human islets, with an RPKM of 15, and was mildly induced by palmitate (Fig. 5*A*). The induction was not detected in independent human islet samples (Fig. 5*B*), but palmitate did induce CREB3 expression in INS-1E cells (Fig. 5*C*). CREB3 silencing (using two independent siRNAs, Fig. 5*C*) markedly sensitized the cells to palmitate-induced apoptosis (Fig. 5*D*). Similarly, efficient CREB3 knockdown in primary rat β-cells enhanced lipotoxicity, nearly doubling palmitate-induced apoptosis (Fig. 5*E* and *F*). This was confirmed in human islets, where CREB3 mRNA knockdown by 67–72% potentiated lipotoxicity in two independent preparations (Fig. 5*G*). CREB3L3 is expressed at lower levels in human islets but was markedly induced by palmitate (Fig. 5*H*); this was confirmed in independent human islet samples by qRT-PCR (Fig. 5*I*). CREB3L3 was also induced by oleate, but inhibited by synthetic ER stressors (Fig. 5*I*), suggesting it mediates an adaptive UPR in response to FFAs, but fails to do so in the face of severe chemical ER stress. Taken together, these data suggest a novel role for CREB3 and CREB3L3 in adaptive β-cell UPR signaling.

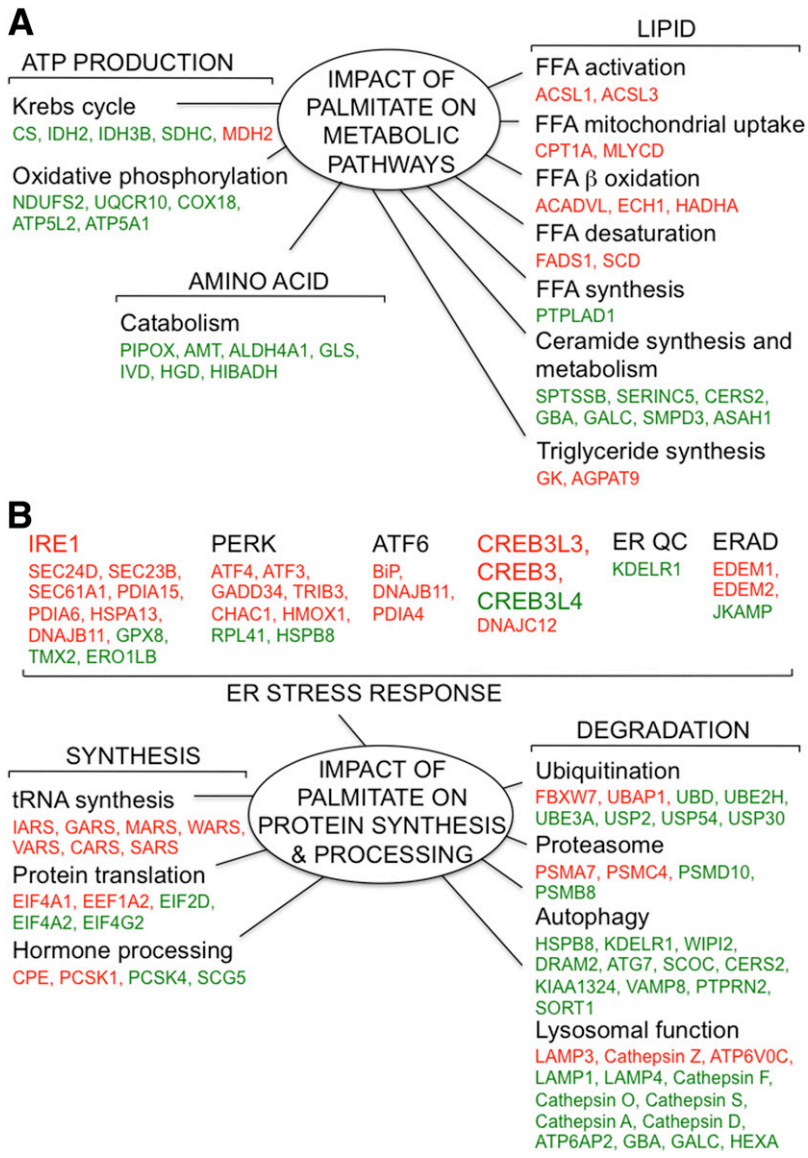


**Figure 3—**Impact of palmitate on human islet metabolic pathways and protein synthesis, ER stress response, and protein degradation pathways. *A*: Manual curation of palmitate-modified human islet transcripts that may affect metabolic pathways pertaining to the Krebs cycle, oxidative phosphorylation, amino acid catabolism, FFA metabolism, ceramide synthesis and metabolism, and triglyceride synthesis. *B*: Manual curation of palmitate-modified human islet transcripts that may affect protein synthesis and processing, pertaining to tRNA synthesis, protein translation, and hormone processing pathways, as well as branches of the ER stress response controlled by IRE1, PERK, and ATF6, ER quality control (QC), and ER-associated degradation (ERAD), ubiquitination, proteasomal degradation, autophagy, and lysosomal function. Upregulated genes are shown in red and downregulated genes in green.

### Proteasomal Function and Autophagy

Palmitate inhibited expression of the ubiquitin-conjugating enzymes UBE2H and UBE3A; the deubiquitinating enzymes USP2, USP54, and USP30; ubiquitin D, a proteasomal degradation signal; and modulated expression of components of the proteasome (Fig. 3*B*).

FFAs have previously been shown to induce autophagosome formation (50,51) and impair autophagic flux in β-cells (52). We confirmed that palmitate induces conversion of microtubule-associated protein 1 light chain 3 (LC3) from its native (I) to the lipidated form (II) in INS-1E cells (Fig. 6*A* and *B*) and human islets (Fig. 6*C* and *D*), resulting in an increased LC3 II-to-I ratio (Fig. 6*B* and *D*) and accumulation of autophagosomes in human β-cells (Fig. 6*E*). The present RNA-seq data identified inhibition of a number of autophagy-related and lysosomal function–related genes that may directly affect lysosome– autophagosome fusion (Fig. 3*B*). Thus, ATG7 and WIPI2 were inhibited, as were the positive regulators of autophagy SCOC, DRAM2, and KIAA1324. ATP6AP2, an accessory protein to the $H^+$-ATPase, and the adaptor-related protein complex subunit AP2M1, both of which contribute to lysosomal acidification, were inhibited. The inhibition of adaptor-related protein complex subunits AP3B1 and AP3M2 and cathepsins F, O, S, A,

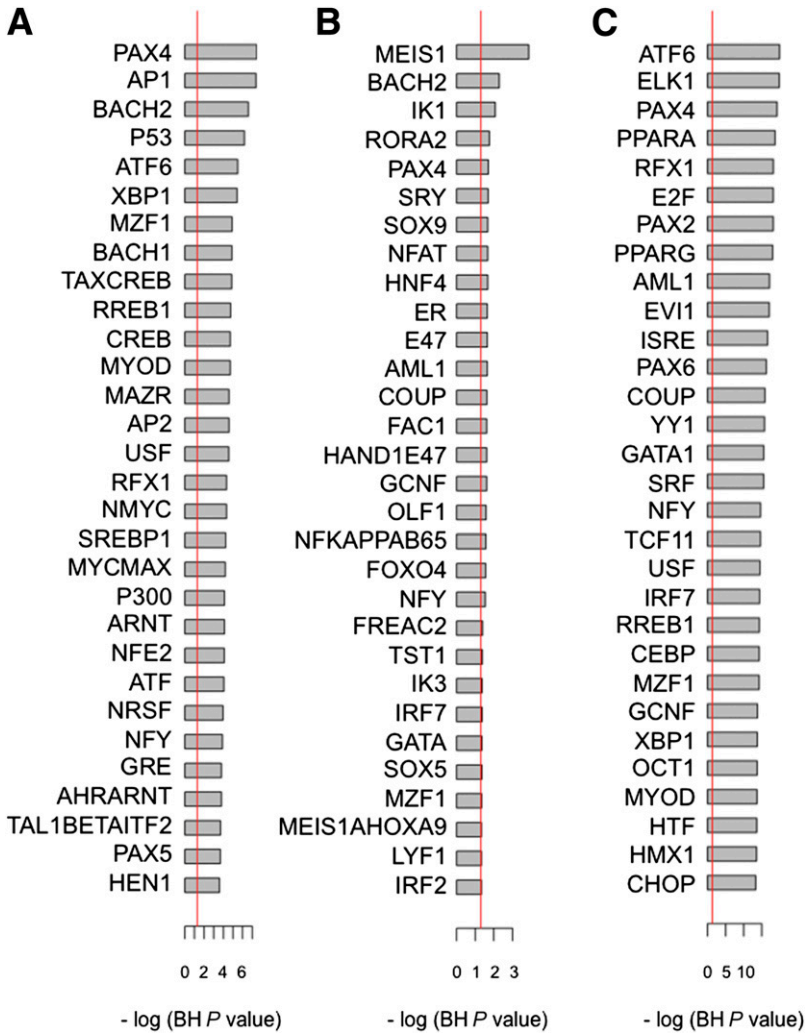


**Figure 4**—DAVID analysis of palmitate-modified genes in human islets. RNA-seq data of transcripts upregulated (*A*) or downregulated (*B*) by palmitate or with modified alternative splicing (*C*) were analyzed for term enrichment against UCSC_TFBS. The length of the bars indicates the significance of the overrepresentation of potential binding sites for the indicated transcription factors in the modified genes, expressed as minus the logarithm of the probability that a set of genes taken at random from the human genome would pop up the same entries. Only the 30 top entries are displayed. The vertical line indicates a probability threshold of 0.05, corresponding to a −log(BH *P* value) of 1.3.

and D may also affect lysosomal function. To examine whether stimulating autophagic flux would protect β-cells, we used the autophagy-enhancing drug carbamazepine (53). Carbamazepine protected INS-1E cells from lipotoxicity in a dose-dependent manner (Fig. 6*F*). In human islets, carbamazepine promoted LC3 II disappearance (Fig. 6*G* and *H*), suggesting increased clearance of autophagosomes (54), and effectively protected against palmitate-induced cell death (Fig. 6*I*).

### Mitochondrial Dynamics and Quality Control

We searched the human islet RNA-seq data for genes involved in mitochondrial movement, biogenesis, fusion-fission, and mitophagy (Supplementary Table 5). Mitochondrial fragmentation, which plays a role in lipotoxic β-cell apoptosis (55), was detected ultrastructurally in palmitate-treated human islets (Supplementary Fig. 5*A*). Genes involved in mitochondrial fission (DNM1L, FIS1, MFF) and fusion (MFN1, MFN2, OPA1) were well expressed in human islets, with a median RPKM of 12, compared with a median expression of 6 RPKM for all human islet transcripts. Palmitate did not modify their expression, suggesting that the mechanism(s) leading to impaired mitochondrial fusion is not transcriptional.

Among the mitochondrial enzymes induced by palmitate was Lon peptidase 1 (LonP1, Supplementary Fig. 5*B*). This $AAA^+$ protease is involved in mitochondrial protein quality control. It degrades misfolded or oxidized proteins and acts as a chaperone in the assembly of protein complexes (56). We confirmed that LonP1 is induced under lipotoxic conditions in rat β-cells (Supplementary Fig. 5*C* and *D*). LonP1 induction by palmitate (57) may occur in response to a stoichiometric imbalance in nuclear- and mitochondrial-encoded proteins.

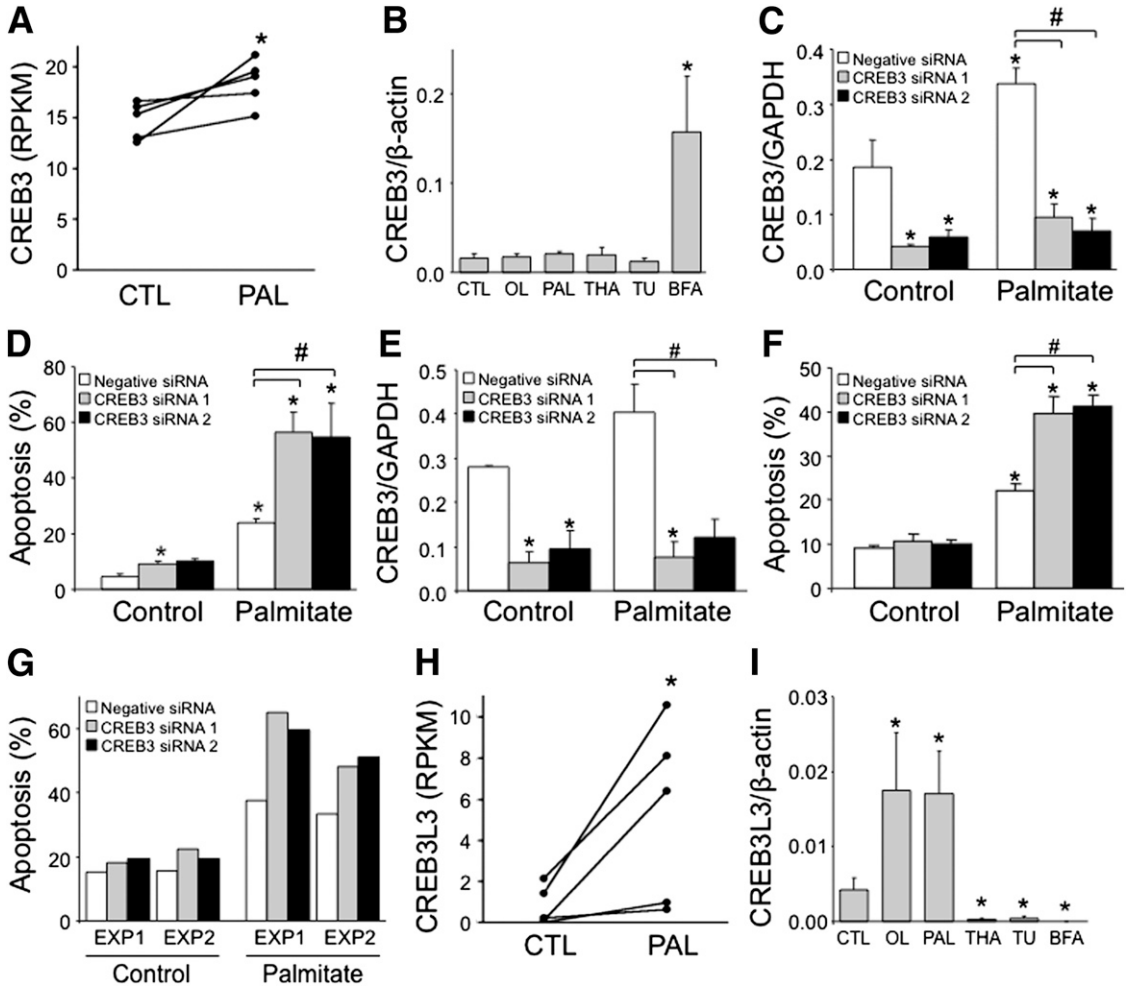


**Figure 5**—Role of novel ER stress transducers in lipotoxic β-cell death. *A*: RNA-seq data of CREB3 expression in five human islet preparations exposed to palmitate for 48 h. *B*: CREB3 mRNA expression assessed by qRT-PCR in human islets exposed to 0.5 mmol/L oleate (OL), 0.5 mmol/L palmitate (PAL), 1 μmol/L thapsigargin (THA), 5 μg/mL tunicamycin (TU), or 0.1 μg/mL brefeldin A (BFA) ($n$ = 3–6). CREB3 mRNA expression assessed by qRT-PCR (*C*) and apoptosis (*D*) in INS-1E cells transfected with control (negative) or two different CREB3 siRNAs and then treated with 0.5 mmol/L palmitate for 16 h ($n$ = 3). CREB3 mRNA expression (*E*) and apoptosis (*F*) in fluorescence-activated cell sorter–purified primary rat β-cells transfected with control or CREB3 siRNAs and then treated with 0.5 mmol/L palmitate for 24 h ($n$ = 3). *G*: Apoptosis in dispersed human islet cells transfected with control or CREB3 siRNAs and exposed to palmitate for 24 h. Individual data for two independent human islet preparations are shown. *H*: RNA-seq data of CREB3L3 expression in five human islet preparations exposed to palmitate for 48 h. *I*: CREB3L3 mRNA expression assessed by qRT-PCR in human islets exposed to ER stressors as in panel *B* ($n$ = 3–6). *$P$ < 0.05 vs. control (CTL) or control cells transfected with negative siRNA; #$P$ < 0.05 as indicated.

### Insulin Secretion

Palmitate is known to inhibit glucose-induced insulin release, and this was confirmed here. Palmitate-treated human islets increased insulin secretion after high glucose stimulation by 1.4 ± 0.1-fold, compared with a stimulation index of 2.1 ± 0.2 for nonexposed islets ($P < 0.01$, Supplementary Fig. 6). We performed a detailed analysis of the effect of palmitate on the expression of human islet genes that modulate insulin secretion mostly at the level of membrane depolarization and $Ca^{2+}$ entry (58). Palmitate inhibited only 2 of the 48 genes (Supplementary Table 6), including the $Ca^{2+}$-sensing receptor, whose activation contributes to human islet insulin secretion (59). Palmitate did inhibit expression of genes involved in ATP production in the Krebs cycle and respiratory chain, including citrate synthase, 2 isocitrate dehydrogenases, and components of complexes I, II, III, and IV and mitochondrial ATP synthase (Fig. 3*A* and Supplementary Table 4). This inhibition, but not transcriptional inhibition of the distal steps of the stimulus-secretion pathway, may contribute to loss of insulin secretion (60,61).

Interestingly, several of the genes implicated in stimulus-secretion coupling have splice variants. For at least some (KCNMA1, $Ca^{2+}$-sensing receptor, and CLCN3), palmitate alters splicing (Supplementary Table 6). Future studies should investigate the functional impact of these changes in splicing.

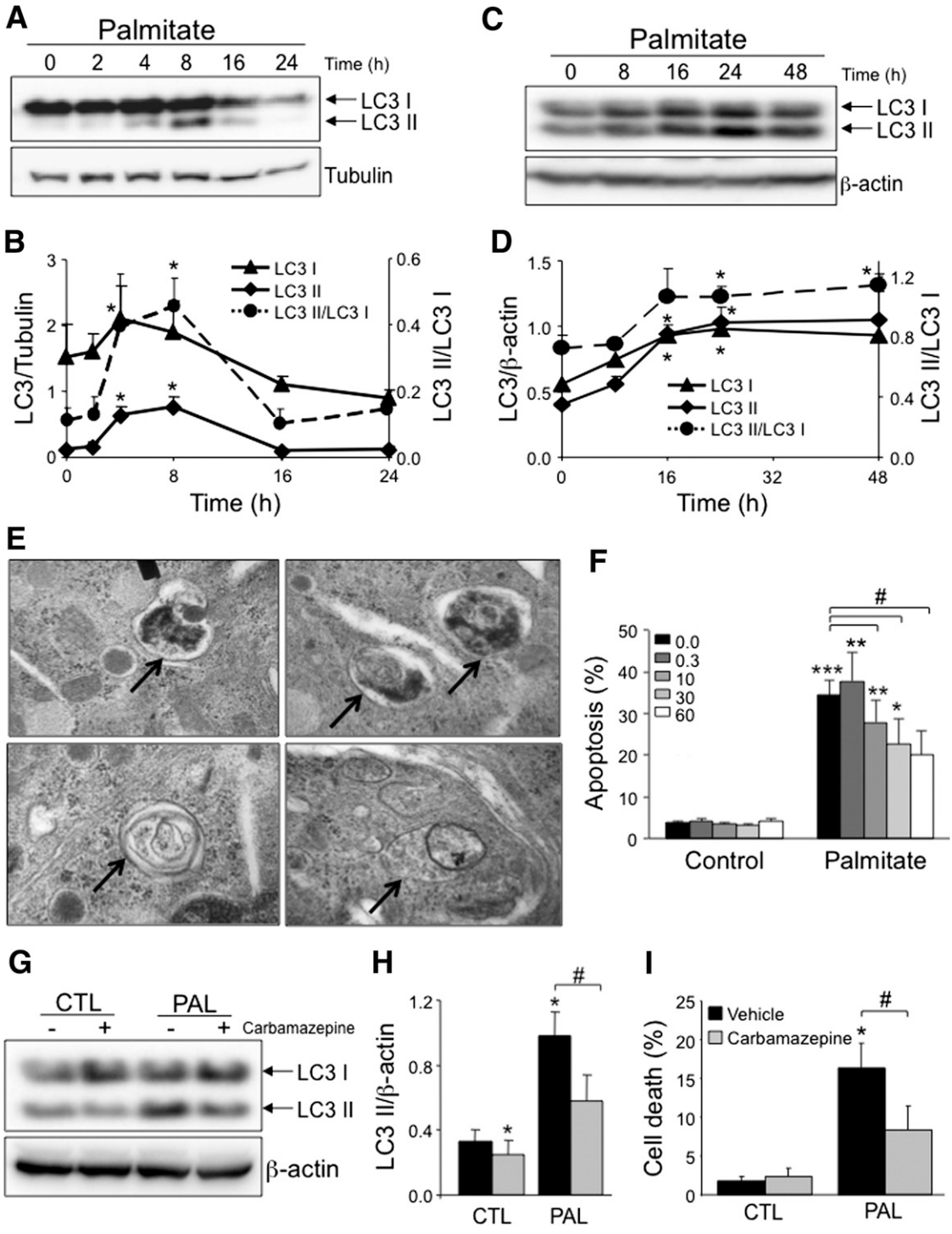


**Figure 6**—Role of autophagy in lipotoxic β-cell death. LC3 conversion in INS-1E cells (*A* and *B*) and human islets (*C* and *D*) exposed to 0.5 mmol/L palmitate (PAL) for the indicated times ($n$ = 3–4). *B* and *D*: LC3 I and II protein expression was quantified by densitometry and normalized to tubulin or β-actin or expressed as the ratio of LC3 II-to-I. *E*: Accumulation of autophagosomes (double membrane organelles containing rough ER and mitochondria and/or partially degraded ER; arrows) in human islets exposed to palmitate for 48 h. *F*: Apoptosis in INS-1E cells exposed to 0.5 mmol/L palmitate alone or in combination with the indicated concentrations (in μmol/L) of carbamazepine for 24 h ($n$ = 5–7). *G* and *H*: LC3 conversion in human islets exposed to 0.5 mmol/L palmitate and/or 30 μmol/L carbamazepine for 24 h ($n$ = 3). *I*: Human islet cell death after 24 h exposure to palmitate and/or carbamazepine ($n$ = 4). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control (CTL); $\#P < 0.05$ as indicated.

### Transcription Factors

Palmitate downregulated PDX1, MAFA, MAFB, PAX4, and NEUROD1, which are important for the maintenance of β-cell function and its differentiated state (Fig. 2). The RNA-seq data also pointed to a mild inhibition of GATA6, a well-expressed transcription factor (RPKM 7, Fig. 7*A*). Heterozygous GATA6 mutations cause pancreatic agenesis and neonatal diabetes (62) and milder phenotypes such as adult-onset diabetes. We confirmed by qRT-PCR that palmitate inhibits GATA6 expression in independent human islet samples and in primary rat β-cells (Fig. 7*B* and *C*). GATA6 was silenced by transfecting rat or human islet cells with two different siRNAs (Fig. 7*D–F*). GATA6 knockdown induced apoptosis under the basal condition and accentuated lipotoxicity (Fig. 7*G–I*). These data point to a novel role for GATA6 in adult β-cells and suggest that its inhibition by palmitate contributes to lipotoxicity. Interestingly, the promoter regions of transcripts that were downregulated by palmitate were enriched in potential GATA binding sites (Fig. 4*B*).

### Cell Death and Inflammatory Responses

Palmitate induced mRNAs encoding pro- and antiapoptotic proteins (Fig. 2 and Supplementary Table 4); for example, palmitate induced GRAMD4, which inhibits the antiapoptotic Bcl-2 protein and promotes Bax translocation to the mitochondria. Some proapoptotic genes were inhibited, including TP53INP1, caspase 2 and 10, and the proapoptotic Bcl-2 family members BMF and BCL2L11, which encodes Bim. The latter is in keeping with our previous findings that Bim does not play a role in lipotoxic β-cell apoptosis (19). Palmitate also inhibited genes with antiapoptotic functions, including c-Flip, DDX17, TM7SF3, DCAF7, ADCYAP1, ANXA4, NMT1, and PRDX6.

TXNIP expression was inhibited by palmitate; this was confirmed by qRT-PCR (Supplementary Fig. 7*A*). High glucose tended to increase TXNIP expression, but palmitate prevented the induction of TXNIP by glucose (Supplementary Fig. 7*A*). TXNIP inhibits insulin secretion and promotes apoptosis; its inhibition indicates it does not mediate lipotoxicity. This is in keeping with an earlier report showing that TXNIP deficiency protects against gluco- but not lipotoxicity (63). Chen et al. (63) also suggested that TXNIP does not mediate thapsigargin-induced β-cell apoptosis. In contrast, a recent report indicated that ER stress induces TXNIP and thereby causes NLRP3 inflammasome activation and interleukin (IL)-1β–driven human islet apoptosis (64).

We previously showed that an IL-1 receptor antagonist does not protect human islets from palmitate (31). Similarly, the IL-1 receptor antagonist did not protect human islets against apoptosis induced by thapsigargin or brefeldin A, whereas it effectively protected against the cytokines IL-1β and interferon-γ (Supplementary Fig. 7*B*). Notably, the NLRP3 inflammasome has very low expression in human islets. The RPKM sum of the five NLRP3 transcripts has a mean value of 0.12, and NLRP3 is not induced by palmitate (0.06 RPKM), one of the priming steps for NLRP3 activation. PYCARD and caspase 1 are expressed (mean RPKM of 7.9 and 4.1, respectively) but not modified by palmitate. These data argue against a proapoptotic role of TXNIP and the inflammasome in human islets facing lipotoxicity or ER stress.

Palmitate upregulated chemokines and cytokines, including IL-6, IL-1A, IL-33, IL-8, CXCL1, and CXCL2 (Fig. 2 and Supplementary Table 4), in line with previous findings (31); the role of these mediators remain to be defined.

### Long Noncoding RNA

Our experimental design and analysis was not directed at long noncoding RNA (lncRNA) discovery. From the RefSeq database, 1,297 of the 3,267 known noncoding RNAs (a global class of noncoding RNAs, including lncRNAs, miRNAs, pseudogenes, unspliced transcripts, etc.) were present in human islets (RPKM $>$ 1). Recently, a large number of previously unknown lncRNA were identified in human islets and β-cells (65). We detected 349 of these 1,128 lncRNA; of these, 9 (2%) were modified by palmitate, showing their responsiveness to metabolic stimuli. The induction of MALAT1, one of the most abundant lncRNA, was confirmed by qRT-PCR (Supplementary Fig. 2).

### Palmitate-Induced Changes in T2D Candidate Gene Expression

Palmitate inhibited expression of TSPAN8, KCNK16, ADCY5, ADRA2A, TP53INP1, CDC123, and PRC1 but induced C2CD4A, ADAMTS9, and SPRY2 (Fig. 1). For one of these, we evaluated the functional consequence of its downregulation. ADCY5 silencing (by 50%) markedly sensitized rat β-cells to apoptosis, basally and after palmitate exposure (Supplementary Fig. 8).

### Palmitate-Induced Alternative Splicing in Human Islets

Of the 212 human islet–expressed splicing factors, 17 were modified by palmitate (Fig. 8*A*). Among these was SRSF3 (Fig. 8*B*). We confirmed by qRT-PCR that palmitate induces SRSF3 in independent human islet samples and in rat β-cells (Fig. 8*C*).

Exposure of human islets to palmitate altered splicing of 574 genes, with 363 and 462 splice variants being up- and downregulated, respectively, using RefSeq annotation (Supplementary Fig. 9*A*). IPA of palmitate-modified splice variants identified "Cell Growth and Proliferation" and "Cell Death" as the main categories (Supplementary Fig. 9*B*). Because RefSeq provides a conservative catalog of splice variants, we reanalyzed the RNA-seq data using the ENCODE-based GENCODE data set (version 16), which provides four-to-fivefold more transcripts. This increased the number of splice transcripts modified by palmitate by more than sixfold, to 3,525, corresponding to 2,858 genes (Fig. 8*D*). Compared with the splicing induced in human islets by cytokines, there was little overlap (14% only, Supplementary Fig. 1*C*), showing a stress-specific

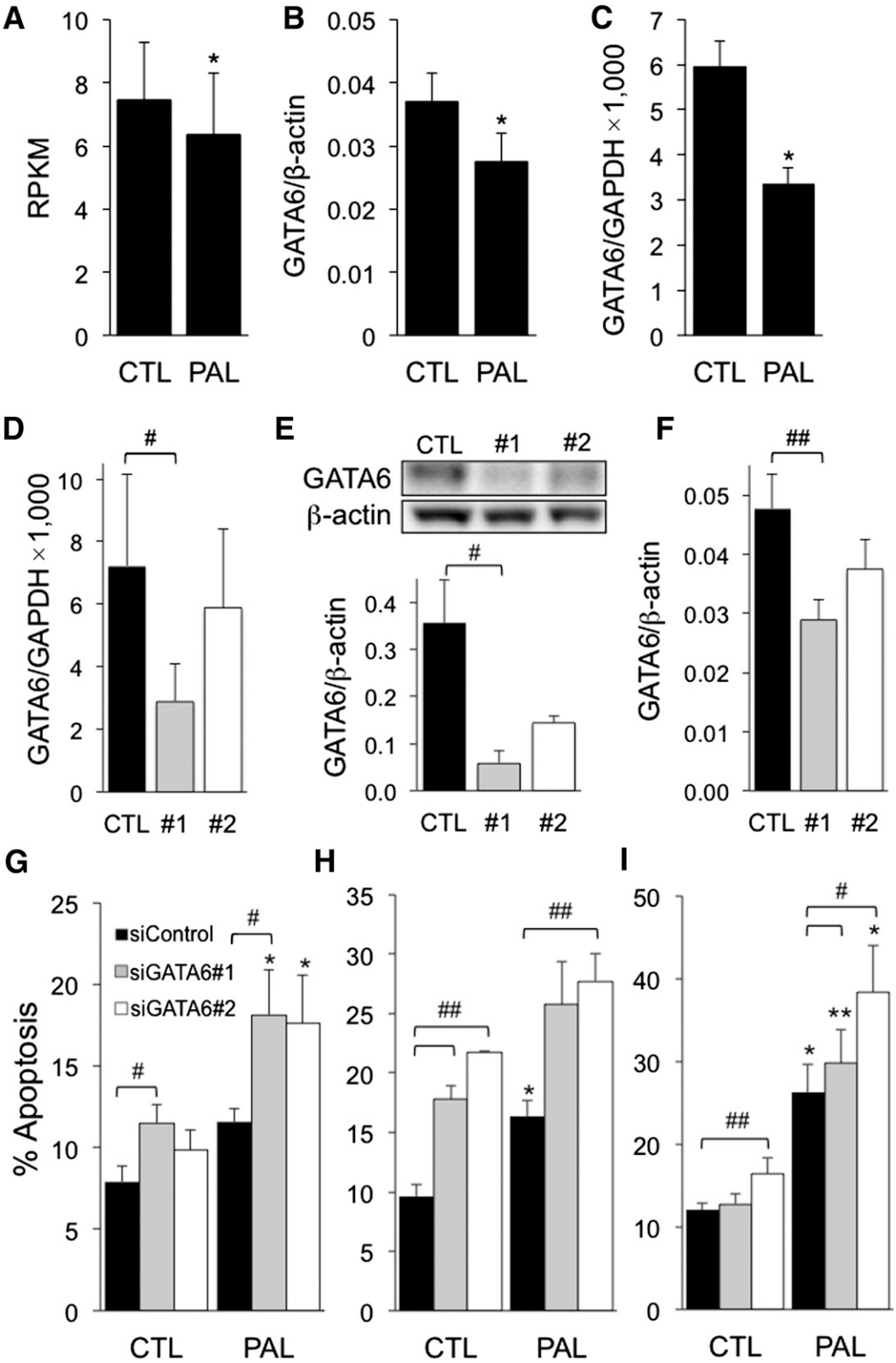


**Figure 7—**GATA6 inhibition by palmitate may contribute to β-cell death. GATA6 expression in five human islet preparations exposed or not (CTL) to palmitate (PAL) for 48 h, measured by RNA-seq (*A*) and confirmed by qRT-PCR (*B*). *C*: GATA6 expression by qRT-PCR in fluorescence-activated cell sorter–purified primary rat β-cells exposed to palmitate for 48 h ($n$ = 4). Primary rat β-cells (*D*), INS-1E cells (*E*), and dispersed human islets (*F*) were transfected with control siRNA (CTL) or two different siRNAs targeting GATA6 (#1 and #2). GATA6 mRNA expression was assessed by qRT-PCR ($n$ = 4–7) (*D* and *F*) and protein expression by Western blot ($n$ = 3) (*E*). Apoptosis in primary rat β-cells (*G*) and dispersed rat (*H*) and human islets (*I*) transfected with control or GATA6 siRNAs and then exposed to palmitate for 24 h ($n$ = 3–6). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated cells; $\#P < 0.05$, $\#\#P < 0.01$ as indicated.

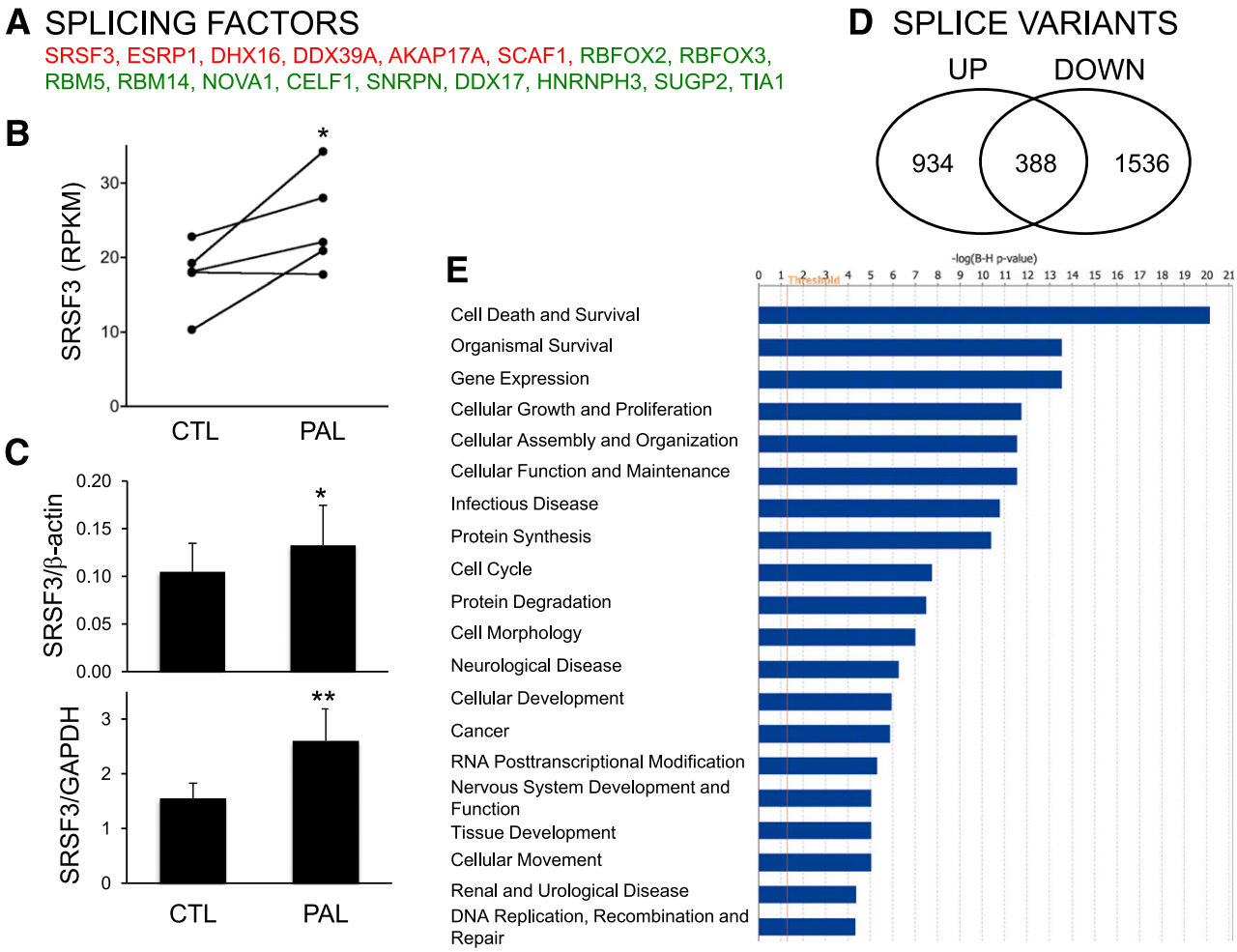


**Figure 8**—Palmitate-induced changes in alternative splicing according to GENCODE annotation. *A*: Palmitate modified the expression of many splicing factors in human islets; upregulated genes are shown in red, downregulated genes in green. *B*: RNA-seq data of SRSF3 expression in five human islet preparations exposed to palmitate for 48 h. *C*: SRSF3 mRNA expression assessed by qRT-PCR in independent human islet samples ($n$ = 5) (*upper panel*) and INS-1E cells ($n$ = 6) (*lower panel*) exposed or not (CTL) to palmitate (PAL) for 48 and 24 h, respectively. $^*P < 0.05$, $^{**}P < 0.01$ vs. untreated cells by ratio $t$ test. *D*: Palmitate exposure led to marked changes in alternative splicing. A total of 1,403 transcripts were significantly upregulated in at least 4 of 5 islet samples and were significantly downregulated in none, and 2,122 transcripts were significantly downregulated using similar criteria. The Venn diagram illustrates the number of genes that have transcripts modified in both directions (intersection) and in only one direction. *E*: IPA of the 3,525 genes with modified splicing. The length of the blue bars indicates the significance of the association between the set of transcripts and the keyword and is expressed as minus the logarithm of the probability that a random set of transcripts from the human genome would be associated with the same keyword. The straight orange line indicates a threshold of 0.05, corresponding to a −log(BH $P$ value) of 1.3. Only the top 20 categories are shown.

splicing response. IPA of palmitate-modified splice variants identified "Cell Death and Survival," "Organismal Survival," and "Gene Expression" as the main categories (Fig. 8*E*). DAVID analysis against UCSC_TFBS identified among the palmitate-modified splice variants enrichment for potential binding sites for the transcription factors ATF6, ELK1, PAX4, and PPARA (Fig. 4*C*).

## DISCUSSION

RNA-seq is a highly reproducible method to interrogate the whole transcriptome and identify novel cellular responses to environmental cues (25). Different from microarrays, it allows detection of high- and low-abundance genes, alternative splice variants, and novel transcripts, identifying 25–75% more genes than conventional arrays and more differentially expressed transcripts (25,66–68). We used it here to map the global response of human islets facing metabolic stress induced by palmitate. The presently used in vitro model of lipotoxicity induces cellular responses in the islets over a 48-h period, compared with slower and more heterogeneous events in vivo. The effects in vivo may also be attenuated by the presence of unsaturated FFAs that decrease the lipotoxicity of saturated FFAs (8). The picture emerging from the analysis of palmitate-treated human islets indicates a complex adaptive response, including upregulation of lipid metabolism and disposal,

paralleled by inhibition of the Krebs cycle and oxidative phosphorylation. There are several signals of cellular stress responses, including cytosolic stress, mitochondrial quality control, and activation of an array of genes regulating the UPR and pathways of apoptosis. There was also inhibition of genes regulating protein degradation and autophagy, which may aggravate the ER stress by preventing disposal of misfolded proteins. The transcriptome data further suggest that palmitate leads to loss of the β-cell differentiated phenotype, with inhibition of key β-cell transcription factors, hormones, and receptors. Some cytokines and chemokines were induced while HLA genes were inhibited. Palmitate also induced changes in the alternative splicing of more than 3,500 transcripts. These data extend significantly beyond previous microarray findings (19–24). Thus, the RNA-seq analysis identified modulation of the transcription factors GATA6, PAX4, CREB3, and CREB3L3 by palmitate.

GWAS have identified more than 60 loci associated with T2D. The present and previous observations (26,27) indicate that >80% of the putative candidate genes are expressed in human islets, and we show here that some are modified by palmitate exposure.

Palmitate inhibited the transcription factors PDX1, MAFA, MAFB, NEUROD1, PAX4, and GATA6, all of which play important roles in β-cell differentiation. Palmitate modification of GATA6 and PAX4 expression has not been previously reported. In the DAVID analysis, GATA and PAX4 binding sites were overrepresented in the promoter region of palmitate-modified genes and transcripts with modified splicing (Fig. 4). These data suggest that palmitate modulates gene networks contributing to lipotoxic loss of β-cell function and survival. However, the data suggest that the β-cell functional impairment is not related to transcriptional inhibition of distal steps of insulin secretion.

A key cellular stress response activated by palmitate in human islets is the UPR (present data). ER stress has been shown to contribute to lipotoxic β-cell death (13,16). What governs the transition from adaptive to fatal ER stress is probably cell specific and remains ill understood. Saturated FFAs elicit marked PERK activity and the resulting eIF2α phosphorylation contributes to β-cell death (13), whereas ATF6 and IRE1 mediate protective UPR signaling (69). A number of structural homologs of ATF6 may play tissue-specific roles. Here we found that CREB3 and CREB3L3 are upregulated by palmitate. CREB3 may promote protective UPR signaling, given that CREB3 silencing markedly sensitized β-cells to lipotoxicity. A better understanding of the (mal)adaptive facets of the ER stress response in β-cells is important in light of the evidence for UPR markers in β-cells from T2D patients (16,17,30) and that T2D drugs modulate the UPR (69).

The present RNA-seq analysis indicates that palmitate inhibits several protein degradation mechanisms, including autophagy and lysosomal function. In line with the present findings, palmitate has been shown to impair autophagic flux in β-cells and thereby contribute to lipotoxicity (52). β-Cells from T2D patients show signs of altered autophagy, including increased autophagic vacuole and autophagosome volume density and reduced LAMP2 and cathepsin B and D expression in T2D islets (51), in keeping with our RNA-seq findings. We used carbamazepine to stimulate autophagic flux (53) and showed marked β-cell protection from lipotoxicity (Fig. 6*F* and *I*). Carbamazepine is an antiepileptic and mood-stabilizing drug. Compared with other antiepileptics and atypical antipsychotics, carbamazepine is associated with lesser diabetes risk, but no study reported protection from diabetes, possibly because it can impair β-cell function (70).

Palmitate modified splicing of 3,525 transcripts, detection of which is missed by conventional microarrays. The alternative splicing is different from that observed after exposure of human islets to proinflammatory cytokines (26), suggesting that different forms of β-cell stress lead to different splicing signatures, probably through the activation of distinct splicing factors.

In conclusion, the present transcriptomic study provides a snapshot of the β-cell responses to conditions that may contribute to T2D pathogenesis. The transcript changes induced by palmitate differ from cytokine-induced stress (26), showing activation of stress-specific signatures in β-cells. The RNA-seq data identify novel players in palmitate-induced β-cell dysfunction and death and indicate several novel areas for investigation in the field.

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**Author Contributions.** M.C., M.I.M., P.M., and D.L.E. designed the experiments. M.C., B.A., G.B., D.A.C., M.I.-E., M.M., J.-V.T., T.G., O.V., I.S., M.B., L.L., L.M., and M.S. performed experiments and analyzed data. M.C. and D.L.E. wrote the manuscript. B.A., G.B., D.A.C., M.I.-E., M.M., J.-V.T., T.G., O.V., I.S., L.M., and M.S. reviewed and edited the manuscript. M.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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