Heliyon

ELSEVIER

PTB and TIAR binding to insulin mRNA 3′- and 5′UTRs; implications for insulin biosynthesis and messenger stability

Rikard G. Fred [a], Syrina Mehrabi [a], Christopher M. Adams [b,1], Nils Welsh [a,*]

[a] *Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden*

[b] *Department of Biological and Medical Mass Spectrometry, Uppsala University, Uppsala, Sweden*

* Corresponding author at: Nils Welsh, Dept. of Medical Cell Biology, Uppsala University, Husargatan 3 Box 571, SE-751 23, Uppsala, Sweden.

E-mail address: Nils.Welsh@mcb.uu.se (N. Welsh).

[1] Present address: Stanford University Mass Spectrometry, Stanford, CA.

Abstract

Objectives: Insulin expression is highly controlled on the posttranscriptional level. The RNA binding proteins (RBPs) responsible for this result are still largely unknown.

Methods and results: To identify RBPs that bind to insulin mRNA we performed mass spectrometry analysis on proteins that bound synthetic oligonucloetides mimicing the 5′- and the 3′-untranslated regions (UTRs) of rat and human insulin mRNA *in vitro*. We observed that the RBPs heterogeneous nuclear ribonucleoprotein (hnRNP) U, polypyrimidine tract binding protein (PTB), hnRNP L and T-cell restricted intracellular antigen 1-related protein (TIA-1-related protein; TIAR) bind to insulin mRNA sequences, and that the *in vitro* binding affinity of these RBPs changed when INS-1 cells were exposed to glucose, 3-isobutyl-1-methylxanthine (IBMX) or nitric oxide. High glucose exposure resulted in a modest increase in PTB and TIAR binding to an insulin mRNA sequence. The inducer of nitrosative stress DETAnonoate increased markedly hnRNP U and TIAR mRNA binding. An increased PTB to TIAR binding ratio *in*

vitro correlated with higher insulin mRNA levels and insulin biosynthesis rates in INS-1 cells. To further investigate the importance of RNA-binding proteins for insulin mRNA stability, we decreased INS-1 and EndoC-βH1 cell levels of PTB and TIAR by RNAi. In both cell lines, decreased levels of PTB resulted in lowered insulin mRNA levels while decreased levels of TIAR resulted in increased insulin mRNA levels. Thapsigargin-induced stress granule formation was associated with a redistribution of TIAR from the cytosol to stress granules.

Conclusions: These experiments indicate that alterations in insulin mRNA stability and translation correlate with differential RBP binding. We propose that the balance between PTB on one hand and TIAR on the other participates in the control of insulin mRNA stability and utilization for insulin biosynthesis.

Keywords: Biological sciences, Cell biology

1. Introduction

Type 2 diabetes is characterized by a relative lack of insulin [1, 2], which may in part be due to an insufficient biosynthesis of the hormone [3]. The amount of insulin synthesized in beta-cells depends on the levels of translatable insulin mRNA and how efficiently the messenger is used for insulin biosynthesis. Due to the high copy number of insulin mRNA in insulin producing cells, alterations in insulin mRNA levels result to a large extent from changes in messenger stability, rather than transcriptional mechanisms [4]. Post-transcriptional control of mRNA stability is primary carried out by RNA binding proteins (RBPs) [5]. RBPs perform a wide array of functions that determine the splicing, stability, localization and translation of the transcripts [6]. Previous work on insulin mRNA has shown that both the 5′- and the 3′-untranslated regions (UTRs) of insulin mRNA are important for glucose-induced insulin biosynthesis, and that the 3′-UTR is especially important for insulin mRNA stability [4, 7, 8]. The polypyrimidine tract binding protein (PTB) is a RNA binding protein that has been shown to participate in pre-mRNA splicing [9], poly-adenylation [10], mRNA transport [11], IRES-mediated translation [12] and mRNA stabilization [13]. In insulin producing beta-cells PTB is known to stabilize the insulin mRNA by binding to a conserved pyrimidine rich sequence within the 3′-UTR insulin mRNA [4]. PTB also binds to the 5′-UTR of the human insulin mRNA which we and others have previously linked to cap-independent translation of insulin [14, 15]. Interestingly, PTB also stabilizes other proteins involved in the secretory pathway of insulin [16]. This supports the idea that specific RBPs can interact with distinct subsets of mRNAs to coordinate gene expression patterns in response to environmental and cellular conditions [17]. In other cell systems, PTB is known to exert its functions on mRNA stability and translation in cooperation with other RNA-binding proteins [18]. This is in line with the notion that the diverse and complex functions performed by the RBP

requires the activities of multiprotein complexes. Thus, it can be hypothesized that PTB together with other proteins are involved in the regulation of insulin mRNA.

The aim of our work was therefore to identify putative RBPs that bind to insulin mRNA and possibly participate in the control of insulin mRNA stability and translation. We demonstrate that a glucose-induced increase in the affinity of PTB and TIAR to the insulin mRNA UTR regions correlated with increased insulin biosynthesis rates. In contrast, a nitric oxide-induced increase in the affinity of hnRNP U and TIAR correlated with decreased insulin biosynthesis. In addition, down-regulation of PTB levels decreased insulin mRNA levels whereas down-regulation of TIAR levels resulted in the opposite. Taken together these experiments indicate that the beta cell utilizes several RNA binding proteins to regulate the stability and translation of insulin mRNA.

2. Materials and methods

2.1. Cell culture

The rat insulinoma cell line INS-1 832/13 was cultured in RPMI 1640 and maintained in a humidified air + 5% CO_2 incubator at 37 °C. The media used for INS-1 832/13 cells was supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol and antibiotics. Human EndoC-βH1, a kind gift from Profs. R. Scharfmann and P. Ravassard, INSERM, Paris, France, cells were cultured in extracellular matrix/fibronectin-coated plates in 5.5 mM glucose Dulbecco's Modified Eagle medium (DMEM) with supplements as previously described [19].

2.2. Human islets

Isolated human islets were provided by Professor Olle Korsgren (the Department of Radiology, Oncology and Clinical Immunology at Uppsala University Hospital, Uppsala, Sweden) through the Uppsala facility for the isolation of human islets from Scandinavian brain-dead individuals, and through the JDRF award 31-2008-416 (ECIT Islet for Basic Research program). The islets were precultured for 3–7 days in CMRL 1066 (ICN Biomedicals, Costa Mesa, CA, USA) supplemented with 10 mM HEPES, 2 mM L-glutamine, 50 μg/mL gentamycin, 0.25 μg/mL fungizone (GIBCO, BRL), 20 μg/mL ciproxfloxacin (Bayer healthcare AG, Leverkusen, Germany) and 10 mM nicotinamide. The islet beta-cell percentage was determined using Newport green staining followed by fluorescence microscopy and beta-cell percentages were routinely 30–60%.

2.3. Treatments

The cells were incubated for 3 h either in 1.67 mM glucose (LG), 11.1 mM glucose (control) 16.7 mM glucose (HG), 11.1 mM glucose + 1 mM IBMX or 11.1 mM glucose + 1 mM DETAnonoate.

2.4. Preparation of cytoplasmic extract

After incubation the INS-1 cells/human islets were washed in cold phosphate buffered saline and resuspended in solution A, a 10 mM HEPES buffer (pH 7.9) supplemented with 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and proteinase and phosphatase inhibitor cocktail (Thermo Scientific). The cells were incubated on ice for 10 min and following a brief centrifugation and again resuspended in solution A before they were lysed with an electric homogenizer. The lysates were then centrifuged for 5 min at 800 x g at 4° C, after which the supernatants were collected. At this point 40 μl of the cytoplasmic extract was saved as total lysate control.

2.5. RNA-oligonucleotide affinity binding

Streptavidin coated Dynabeads were washed in the recommended washing and binding (B/W) buffer. The beads were then divided into separate Eppendorf tubes (300 mg/group) and 1 nmol of biotin labeled RNA oligonucleotide was added. The oligonucleotides used were:

Rat

5′ short: 5′-Biotin-ACCAUCAGCAAGCAGGUCAUUGUUCCAAC-3′

5'long:5′-GCUACAAUCAUAGACCAUCAGCAAGCAGGUCAUUGUUC-CAACAUGGCCCU-Biotin-3′

3′ short: 5′-Biotin-UCCACCACUCCCCGCCCACCCCUCU −3′

3'long:5′-Biotin- CCACCACUCCCCGCCCACCCCUCUGCAAU-GAAUAAAGCCUUUGAAUGAGC-3′

Human

5′ short: 5′-Biotin-GGCCAUCCAGCAGAUCACUGUCCUUCUGCC-3′

5'long:5′-Biotin-AGAGGCCAUCAAGCAGAUCACUGUCCUUCUGC-CAUGGCCCU-3′

3′ short 1: 5′-Biotin-CGCAGCCCCCCACCCGCCGCCUCCU-3′

3′ short 2: 5′-AGGUGGGGCAGGUGGAGCUG-3′

For control, a scrambled non-specific oligonucleotide was used:

Ctrl: 5′-Biotin-GCGAGGAUACGAUAGGCGCAUACAGAAGUA-3′

The tubes were slowly rotated for 30 min at 4° C. After binding of the oligonucleotides the beads were again washed in W/B buffer and resuspended in 400 µl of the cytoplasmic extract. The incubation took place for 60 min at 4° C in the presence of RNAguard. After incubation the beads were washed three times in solution A and resuspended in 1x SDS-sample buffer. To elute the proteins the beads were boiled for 5 min in the SDS-sample buffer.

2.6. 1-D PAGE and digestion

Eluted proteins were separated using 9% one-dimensional SDS-PAGE gels. The gels were fixated in 40% (v/v) ethanol and 10% (v/v) acetic acid, after which the protein bands were visualized using colloidal Coomassie staining. The protein bands specific to the sample lane (i.e. not present in control lane) were excised and samples were in-gel reduced, alkylated, and digested with modified sequence-grade trypsin (Promega, Madison, WI) as previously described [20]. Samples were vacuum-centrifuged and reconstituted prior to analysis in 12 µl of HPLC water containing 0.1% TFA (Sigma).

2.7. Nanoflow LC/MS/MS

All experiments were performed on a 7-Tesla hybrid lin0ear ion trap Fourier transform mass spectrometer (LTQ FT, Thermo Electron, Bremen, Germany) using a modified nanoelectro-spray ion source (Proxeon Biosystems, Odense, Denmark). The HPLC setup consisted of a solvent degasser, nanoflow pump, and thermostated microautosampler (Agilent 1100 nanoflow system). A 15-cm fused silica emitter (75-µm inner diameter, 375-µm outer diameter; Proxeon Biosystems) was used as analytical column. The emitter was packed in-house with a methanol slurry of reverse-phase, fully end-capped Reprosil-Pur C_{18}-AQ 3-µm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using a pressurized "packing bomb" operated at 50–60 bars (Proxeon Biosystems). Mobile phases consisted of 0.5% acetic acid and 99.5% water (v/v) (buffer A) and 0.5% acetic acid and 10% water in 89.5% acetonitrile (v/v) (buffer B). 8 µl of prepared peptide mixture was automatically loaded onto the column over 20 minutes at 5% buffer B, flow rate of 500 nl/min followed by a 90-min gradient from 5 to 45% buffer B at a constant flow rate of 200 nl/min. MS analysis was performed in data-dependent acquisition (DDA) mode in which the mass spectrometer automatically switches between a high resolution survey scan (resolution @ = 100,000; m/z range, 200–1600). The precursor scan was followed by MSMS fragmentation using the complementary techniques of ECD and CAD [20, 21] on the two most intense precursor ions identified in the MS1 scan event.

2.8. Peptide and modification identification

Acquired RAW files were converted to dta files using Extract msn through BioWorks Browser (Thermo Electron), and complementary pairs were identified as previously described [22]. Peptides were identified by searching against the NCBI (National Center for Biotechnology Information) database using the Mascot search engine. Searches were performed permitting 2 trypsin miscleavages and the mass tolerance for monoisotopic peptide identification was set to 10 ppm and ±0.02 Da for fragment ions. The instrument setting was "ESI-FTICR". The peptide mass tolerance, mass accuracy window for fragment ions, and enzyme specificity as well as the instrument settings were kept unchanged. Parsing of data and statistical analysis of the search results reported by Mascot were performed using the open-source software MSQuant [21].

2.9. SDS-PAGE and immunoblotting

Protein samples in SDS-sample buffer were separated on SDS-PAGE. Proteins were electrophoretically transferred to Hybond-P filters (Amersham Biosciences), which were then blocked for one hour using a 5% bovine serum albumin (BSA) protein solution. The filters were probed with primary antibodies for PTB, hnRNP L, hnRNP U, TIAR and ERK. The horseradish peroxidase conjugated anti-mouse, anti-rabbit or anti-goat antibodies were used as secondary antibodies and the immunodetection was performed as described for the ECL immunoblotting detection system by Amersham Biosciences, using the Kodak Image station 4000MM. The resulting bands were quantified using Kodak Digital Science ID software.

2.10. siRNA transfection

For siRNA transfection of INS-1 cells, we used 60 nM of a pre-designed siRNA oligonucleotide directed against PTB with the antisense sequence 5′-AUUUACA-CUCUUCCAUGACtt-3′ (Dharmacon). The siRNA against TIAR was also purchased from Dharmacon (LOC361655 siGENOME SMARTpool). To introduce the siRNAs into the cells we used Lipofectamine (Invitrogen) according to the manufacturer's recommendation. The control consisted of 60 nM of non-targeting siRNA (Dharmacon). The transfection took place for 3 h after which the cells were cultured for 24, 48 or 72 h in complete culture medium. All experiments were run in duplicate and after harvest the cells were either used for semi-quantitative real-time PCR or Western blot. For siRNA transfection of EndoC-βH1 cells Dharmafect I (Dharmacon) was used according to the instructions of the manufacturer. Human smartpool siGENOME siRNA sequences directed against PTB and TIAR, and used at 60 nM, were from Dharmacon.

2.11. Total RNA isolation and cDNA synthesis

Cells were washed once with phosphate-buffered saline and 200 µl of UltraSpec RNA Isolation Solution (BioTecx Laboratories) was added to each sample. Total RNA was extracted according to the instructions of the manufacturer.

The cDNA synthesis was performed using iScript Reverse Transcription Supermix for RT-qPCR (Bio Rad). The synthesis reaction was terminated by heating at 95 °C for 5 min after which the samples were stored at −20 °C. Before the cDNA samples were analyzed by real-time PCR, they were purified using the Viogene PCR-M Cleanup System. All steps were performed according to manufacturers recommendations.

2.12. Semi-quantitative real time PCR

The real time PCR was performed on a Light Cycler II instrument (Roche) using the SYBR Green Taq Readymix (Roche). This value was then normalized against the value for β-actin, GAPDH and G6PDH. Primer sequences are available upon request.

2.13. Insulin and total protein biosynthesis

INS-1 cells were incubated for 3 h in 200 µl sterile Krebs-Ringer bicarbonate buffer supplemented with 10 mM HEPES (Sigma) (KRBH), 2 mg/ml BSA and 50 µCi/ml L-[3,4,5-^{3}H] leucine (GE Healthcare Life Science, Chalfont St. Giles, Buckinghamshire, UK). After incubation, and incorporation of radioactive leucine, the cells were washed once in PBS, harvested using a rubber policeman and homogenized by sonication (22 kHz, 50 W) for 10 s in 200 µl of a 50 mM glycine buffer (pH 8.8) supplemented with 2.5 mg/ml BSA. For insulin analysis, four 10 µl aliquots of each sample were transferred to Ellerman tubes and mixed with 100 µl 50 mM glycine buffer (pH 8.8) supplemented with 2.5 mg/ml BSA and 0.1% Triton X–100, and in duplicates supplied with 10 µl of guinea pig anti-human insulin serum (Chemicon International, Temecula, CA, USA) or 10 µl normal guinea pig serum (Harlan Sera-lab, loughborough, Leicestershire, UK). The tubes were mixed and incubated for 1 h in room temperature. 100 µl protein A-Sepharose (Amersham-Pharmacia Biotech, Uppsala, Sweden) were added and the tubes tumbled at room temperature for 15 min and then centrifuged for 1 min at 200 x g. The Sepharose beads were washed twice in 500 µl buffer and subsequently resuspended in 500 µl 1 M acetic acid with 2.5 mg/ml BSA.

For total protein analysis, two 10 µl aliquots of each sample were transferred to Ellerman tubes, to which 250 µl 50 mM glycine buffer (pH 8.8) supplemented with 2.5 mg/ml BSA and 250 µl of 10% trichloroacetic acid were added. The tubes were vortexed and then centrifuged for 2 min at 14000 x g. After removal of the

supernatant the precipitate was dissolved in 500 µl 0.15 M NaOH. To determine the radioactivity, the samples were transferred to scintillation vials together with 4 ml Ultima Gold scintillation fluid and analyzed with liquid scintillation spectrometry (Wallac System 1409) using external standardization.

2.14. Immunofluorescence

INS-1 832/13 ells were cultured overnight on poly-L-lysine treated coverslips and treated with thapsigargin (200 nM) for different time periods. Cells were then fixated in 4% paraformaldehyde for 10 min, permeabilized for 10 min in PBS + 2% BSA and 0.5% Triton X–100, and then and blocked for 1 h in PBS + 2% BSA. Samples were incubated with TIAR or Dcp1A (dilution respectively 1:100 and 1:200, Santa Cruz Biotechnology) antibodies for 1 h at room temperature. Secondary anti- goat or anti-mouse antibodies (dilution respectively 1/1000 and 1:500; Santa Cruz Biotechnology) were then applied for 1 h at room temperature in the dark.

2.15. Fluorescence microscopy

All images were acquired a Nikon Eclipse TE2000-E laser-scanning confocal microscope using the x60 objective lens. The fluorescence was excited with 488 nm for the green fluorescence and 594 nm for red fluorescence. DAPI staining was used for visualization of cell nuclei.

3. Results

3.1. Identification of proteins that bind to the 3′-UTR of insulin mRNA *in vitro*

To our knowledge, PTB, human antigen D (HuD) and protein-disulfate isomerase are the only RBPs known to associate with insulin mRNA UTR-sequences [4, 23, 24]. To identify other proteins that participate in the control of insulin mRNA stability and translation, a biotinylated RNA oligonucleotide corresponding to nucleotides 392–417 of rat insulin I mRNA was incubated with cytoplasmic extracts from INS-1 cells. As control for unspecific binding we used a scrambled oligonucleotide with the same nucleotide composition. The Dynabead purified proteins were separated on polyacrylamide gels and stained with Coomassie blue (Fig. 1). Bands specific for the insulin mRNA sequence (indicated by arrows) were excised, in-gel digested and analyzed by ultra-high resolution mass spectrometry. The subsequent analysis identified a number of proteins (Fig. 1), including, but not limited to: hnRNP U, hnRNP K, hnRNP L, PTB, polyC-binding protein (PCBP) 1, 2, 3 and TIAR. The mass spectrometry results were confirmed by immunoblotting using commercially available antibodies against PTB, hnRNP U, hnRNP L and TIAR, and these RBPs were chosen for further investigation. HnRNP U is an 120
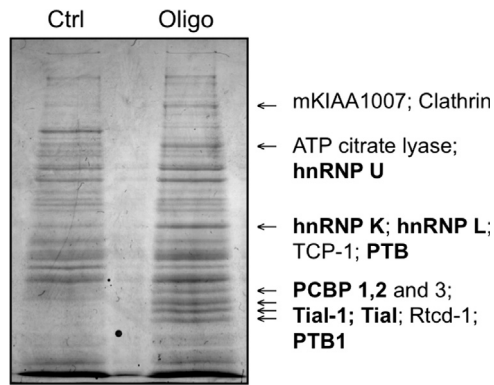
Fig. 1. Identification of proteins that bind to the 3′-UTR of insulin mRNA *in vitro* by mass spectrometry. Cytoplasmic extracts from human islets and INS-1 cells were incubated with streptavidin coated Dynabeads carrying a scrambled RNA oligonucleotide (Ctrl) or an RNA oligonucleotide corresponding to the pyrimidine rich sequence of the insulin 3′-UTR (denoted as 3′ short in Fig. 2). Bound proteins were eluted and separated on polyacrylamide gels that were subsequently fixed and stained with coomassie blue. Specific bands were excised and the proteins identified by Fourier Transform Mass Spectrometry. Identified proteins are shown next to their corresponding bands. Proteins in bold were chosen for further investigation.

kDa RNA/DNA-binding protein that has many functions, for example transcriptional regulation [25], nuclear matrix/scaffold attachment [26] and RNA processing [27]. HnRNP L is a splicing factor that in addition to controlling alternative splicing [28] also influences translation [29] and polyA shortening [30]. TIAR is an RNA binding protein that primarily promotes translational arrest and relocalization of mRNAs to p-bodies and stress granules [31], but also apoptosis [32].

3.2. *In vitro* affinity of PTB, hnRNP U, hnRNP L and TIAR to sequences within the 3′- and 5′-UTRs of insulin mRNA

Both the 3′-UTR and the 5′-UTR of the insulin mRNA are important for control of stability and translation [7]. To further elucidate which parts of the 3′- and 5′-UTRs to which different RBPs bind, we designed 5′- and 3′-UTR sequences that consisted only of the core pyrimidine rich sequence, or UTR-sequences which contained both the pyrimidine rich sequence and the surrounding non-pyrimidine rich sequences (Fig. 2A). In addition, based on a predicted secondary structure (mfold, version 3.5) in which the two insulin mRNA UTRs interact with each other (Fig. 2B), we constructed a sequence consisting of both a long 3′- and a long 5′-UTR, so that intramolecular 5′ to 3′ binding and interaction achieved. The rationale for this is that RBPs may differentiate between single and double stranded mRNA structures upon binding *in vivo*. The different RNA oligonucleotides were allowed to incubate with INS-1 cell extracts and RBP binding was assessed by immunoblot analysis (Fig. 3). The results show that hnRNP U bound to all RNA
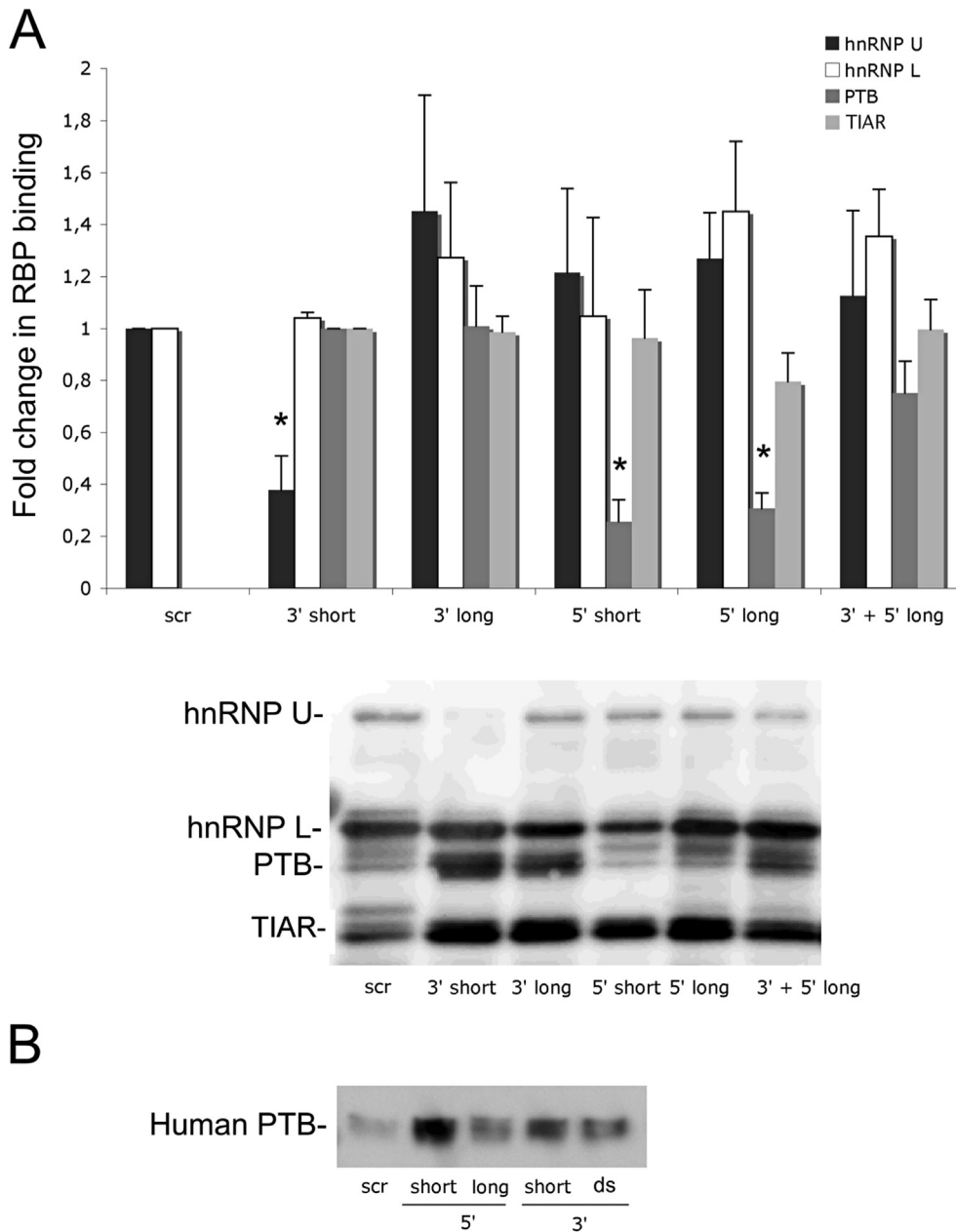
A

B

Fig. 2. RNA oligonucleotides corresponding to specific 3′- and 5′-UTR sequences of the human and rat I insulin mRNA. In (A), the different RNA-oligonucleotides used in the Dynabead pull down experiments are shown. Oligonucleotides representing different regions of the human and rat 3′- and 5′-UTRs were biotinylated so that RNA binding proteins could be affinity purified using streptavidin coated dynabeads. Boxes represent pyrimidine rich sequences with potentially regulatory roles for mRNA stability and translation. The numbering of the first and last nucleotides corresponds to their position on the mRNA according to their NCBI accession number, NM_000207.2 and NM_019129.3 respectively. In (B), a simplified representation of the predicted interaction between the two long rat oligonucleotides is shown.

oligonucleotides except the short 3′-UTR. This suggests that in the case of hnRNP U, some other protein, possibly PTB, blocks the binding to the short pyrimidine rich 3′-UTR oligonucleotide. hnRNP L bound all oligonucleotides, including the scrambled control, with the same affinity, indicating sequence non-specific binding. Interestingly, hnRNP L binding to the short 3′-UTR was not blocked by PTB. Furthermore, PTB binding was very strong to both 3′-UTR oligonucleotides and weaker to the two 5′-UTR oligonucleotides. The weak binding to the 5′-UTR may be explained by the lower content of pyrimidines of the 5′-UTR of rat insulin mRNA as compared to that of the 3′-UTR and the 5′-UTR of human insulin mRNA (Fig. 2A). TIAR bound all insulin mRNA oligonucleotides with similar affinity. The finding that TIAR binding to the short 3′-UTR was not blocked by PTB indicates that the two RBPs do not compete for the same binding site, unlike PTB and hnRNP U. RBP binding to the combination of the long 5′-UTR and the long 3′-UTR was similar to that of only the 3′-UTR.

The binding of PTB in human islet extracts to RNA oligonucleotides with sequences from human 3′- and 5′-UTR of insulin mRNA (Fig. 2A) was also studied. Due to limited availability of human islet material and low signal intensities in immunoblot analysis, we could not study hnRNP L, hnRNP U and TIAR binding in human samples. To see whether PTB prefers dsRNA or ssRNA,

Fig. 3. *In vitro* affinity of RNA binding proteins to oligonucleotides corresponding to specific 3′- and 5′-UTR sequences of rat (A) and human (B) insulin mRNA. RNA binding proteins from INS-1 cells were affinity purified using Dynabeads, separated by SDS-PAGE and analyzed by immunoblotting. The oligonucleotides used were those described in Fig. 2 and in one group (3′ + 5′ long) both the 3′ long and the 5′ long oligonucleotides were loaded on the beads so that complexes between the two oligonucleotides could be formed (Fig. 2B). In (A), protein bands for hnRNP U and hnRNP L were normalized against the signal from a scrambled control oligonucleotide (scr) while PTB and TIAR were normalized against the signal from the short oligonucleotide corresponding to the 3′-UTR of the rat insulin mRNA. The lower panel in A shows a representative blot with all four proteins. In (B), the PTB signal from a corresponding experiment using human islets is shown. Instead of a longer 3′-UTR oligonucleotide a non-biotinylated complementary oligonucleotide was used to mimic double stranded

we used a RNA-oligonucleotide with two short antiparallel 3′-UTR oligonucleotides forming a dsRNA molecule. In two independent experiments we observed that human PTB binds with high affinity, not only to the 3′-UTR of insulin mRNA, but also to the 5′-UTR (Fig. 3B). The binding to the long and the short 5′-UTR oligonucleotides may occur via the pyrimidine rich region of the short 5′-UTR oligonucleotide. The binding of PTB to single stranded and double stranded 3′-UTR was comparable (Fig. 3B). Taken together these results suggest that PTB binds to the pyrimidine rich sequences of both the 3′- and the 5′-UTR in human insulin mRNA and that PTB and hnRNP U compete for binding to the same pyrimidine rich sequence of the rat insulin mRNA 3′-UTR. HnRNP L and TIAR both bind the 3′- and 5′-UTRs at sites different from those for PTB. Finally, RBP binding patterns are not affected by double stranded RNA structures.

3.3. Effects of high glucose, IBMX and DETAnonoate on RBP binding to insulin mRNA 3′- and 5′-UTR oligonucleotides

It is possible that external factors modulate insulin production by altering RBP binding to insulin mRNA. Glucose is a well-known stimulator of insulin mRNA stability and translation [33, 34]. The cAMP raising compound IBMX has been reported to promote PTB translocation from the nucleus to the cytosol, thereby stimulating insulin production [16]. DETAnonoate promotes nitrosative stress and decreased insulin production [35]. To investigate whether the affinities of the aforementioned RBPs to insulin mRNA 3′- and 5′-UTRs are affected by treatments that both stimulate and decrease insulin production, the long 3′- and 5′-UTR oligonucleotides were incubated with protein extracts from INS-1 cells incubated for 180 min with 20 mM glucose, 1 mM IBMX or 1 mM DETAnonoate. We normalized the RNA binding of PTB, hnRNP U and TIAR to that of hnRNP L, as hnRNP L binding was non-specific (Fig. 3) and unaltered by the different treatments (data not shown). The results show that the binding of PTB to the 3′ + 5′-UTRs was modestly increased in response to high glucose (Fig. 4). This finding is consistent with previous studies indicating a stabilizing role of PTB for insulin mRNA [4]. IBMX or DETAnonoate did not affect PTB binding activity. HnRNP U binding was not modulated by high glucose or IBMX, but clearly stimulated by DETAnonoate. Both high glucose and DETAnonoate promoted increased binding of TIAR to the insulin mRNA UTRs. Thus, high glucose promoted a modest increase in PTB and TIAR insulin mRNA binding, whereas nitrosative stress stimulated markedly hnRNP U and TIAR binding.

RNA. The blot is representative for two experiments. Results in A are means ± SEM for 4 independent experiments and * denotes $p < 0.05$ using Student's paired t-test when comparing vs. corresponding control. Full images can be found in Supplemental Fig. 3.
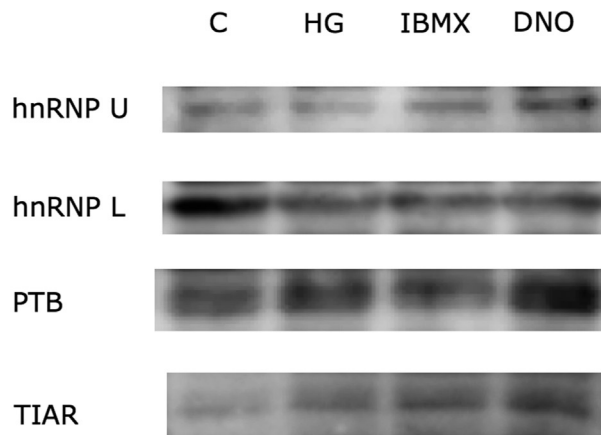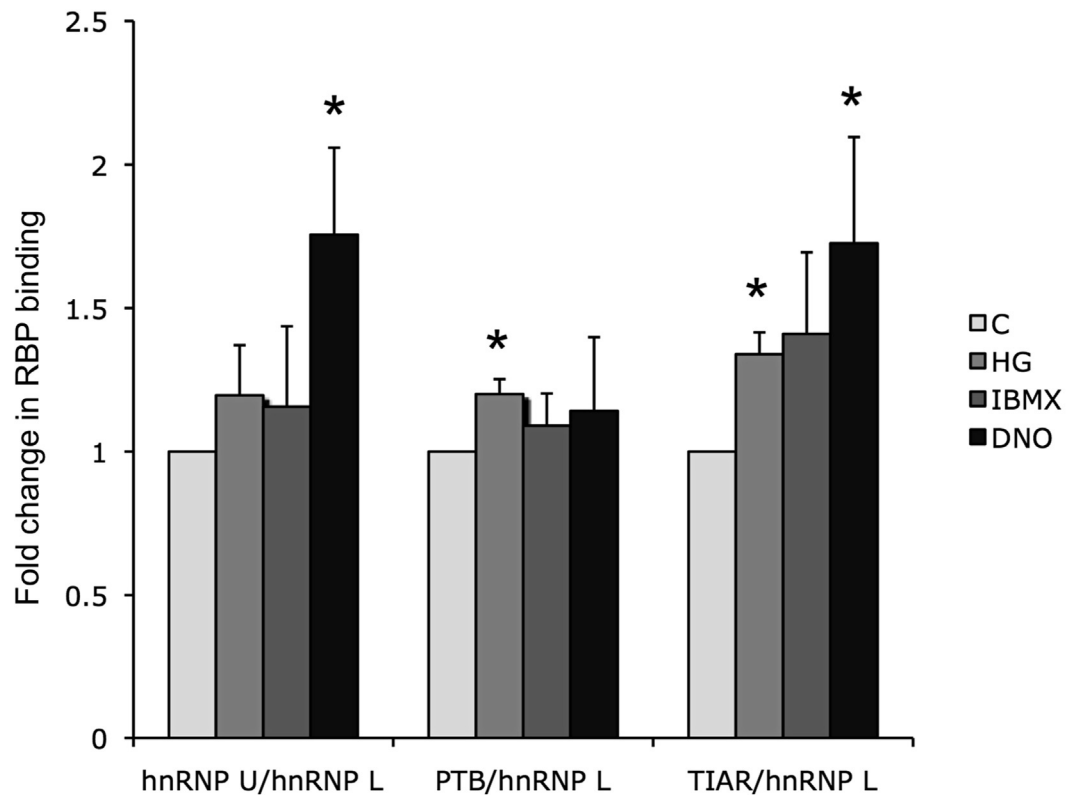
Fig. 4. Effects of high glucose, IBMX and DETAnonoate on the affinity of RNA binding proteins to oligonucleotides representing specific 3′- and 5′-UTR sequences of insulin mRNA in INS-1 cells. INS-1 cells were cultured in RPMI 1640 medium with 1.67 mM glucose (control; C), 16.7 mM glucose (high glucose; HG), 1 mM IBMX and 1 mM DETAnonoate (DNO) for 2 h. Cytoplasmix extracts from the cells were incubated with beads carrying both the long 3′- and the long 5′-UTR oligonucleotides, and the eluted proteins were analyzed by SDS-PAGE and immunoblotting. The affinity of hnRNP U, PTB and TIAR was first normalized against hnRNP L, and then normalized against the control. The lower panel in A shows a representative blot with all four proteins. The image is acquired after blotting for hnRNP U, hnRNP L, PTB and TIAR in sequence, thus showing the correlation from a single

http://dx.doi.org/10.1016/j.heliyon.2016.e00159

3.4. Effects of glucose, IBMX and DETAnonoate on insulin mRNA levels and insulin and total protein biosynthesis rates in INS-1 cells

To correlate the insulin mRNA binding results to changes in the levels of insulin mRNA and the translation of the insulin mRNA we treated the INS-1 cells as above and performed semi-quantitative real-time RT-PCR and insulin and total protein biosynthesis determinations. The results show that incubation in the presence of a high glucose concentration for 3 h augmented insulin mRNA levels as compared to a low glucose concentration (Fig. 5A). Insulin mRNA levels were decreased by IBMX and DETAnonoate (Fig. 5A). Also insulin biosynthesis rates were increased by high glucose as compared to the low glucose group (Fig. 5B). Compared to the 11.1 mM glucose control group both IBMX and DETAnonoate down-regulated insulin biosynthesis (Fig. 5B). The total protein biosynthesis, however, was only decreased by the DETAnonoate treatment (Fig. 5C). These observations indicate that a moderate increase in PTB and TIAR binding to insulin mRNA, promoted by high glucose, is associated with enhanced insulin production, whereas enhanced TIAR and hnRNP U binding, stimulated by nitrosative stress, is associated with decreased insulin production.

3.5. Downregulation of PTB and TIAR in INS-1 and EndoC-βH1 cells leads to lower and higher insulin mRNA levels, respectively

To further substantiate the notion that PTB and TIAR control insulin mRNA stability in opposite directions we down-regulated the two RBPs by siRNA treatment. INS-1 cells were transfected using Lipofectamine and the cells were incubated for 48 or 72 h after which they were harvested for immunoblot analysis and semi-quantiative real-time PCR. The results show that PTB was significantly decreased after 48 h of incubation (Fig. 6A) and that TIAR was downregulated after 72 h (Fig. 6C). These effects were paralleled by increased and decreased insulin mRNA levels, respectively (Fig. 6B, D). Also in the human EndoC-βH1 cells did downregulation of PTB and TIAR (Fig. 7A) result in decreased and increased insulin mRNA levels, respectively (Fig. 7B). Thus, the stability of insulin mRNA seems to be oppositely regulated by PTB and TIAR levels.

experiment. The lower panel shows representative blots for all four proteins. Results are means ± SEM for 3–4 independent experiments and * denotes p < 0.05 using Student's t-test when comparing vs. corresponding control. Full images can be found in Supplemental Fig. 4.
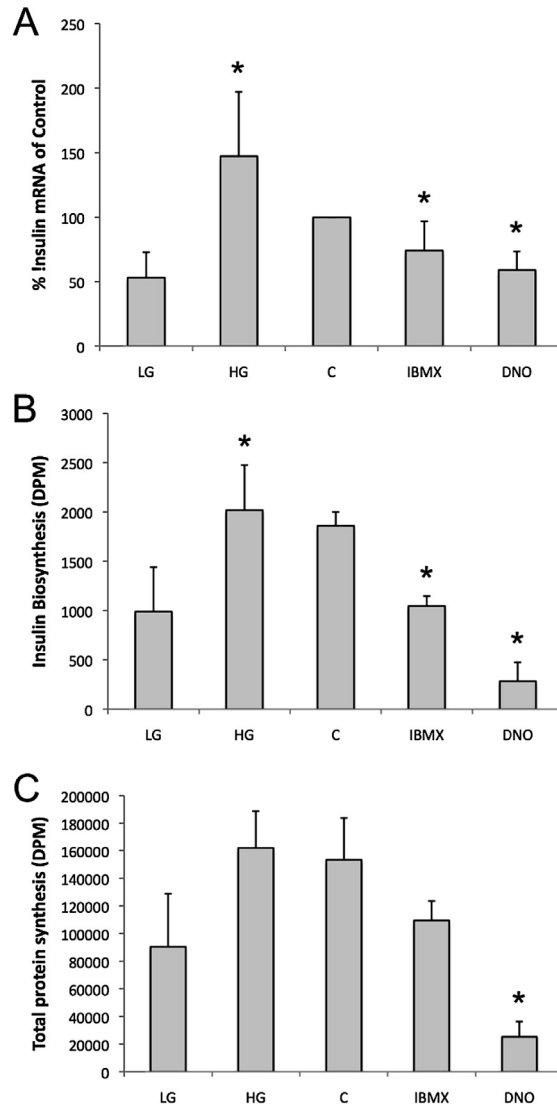
Fig. 5. The effects of glucose, IBMX and DETAnonoate on insulin mRNA levels (A) and insulin (B) and total protein (C) biosynthesis in INS-1 cells. (A) INS-1 cells were cultured for 3 h in RPMI 1640 medium with 1.67 mM glucose (LG), 16.7 mM glucose (HG), 11.1 mM glucose (C), 11.1 mM glucose and 1 mM IBMX (IBMX) or 11.1 mM glucose and 1 mM DETAnonoate (DNO) and were analyzed using real time PCR. Insulin mRNA was normalized against GAPDH and expressed as percent of control. Bars are means ± SD for 9 observations. * denotes p < 0.05 using Student's t-test when comparing HG to LG and IBMX and DNO to C. (B) INS-1 cells were labeled with ^{3}H-leucine for three h in a KRBH buffer containing BSA and 1.67 mM glucose (LG), 16.7 mM glucose (HG), 11.1 mM glucose (C), 11.1 mM glucose and 1 mM IBMX (IBMX) or 11.1 mM glucose and 1 mM DETAnonoate (DNO). Insulin was immunoprecipitated with guinea pig anti-insulin antibodies and protein A sepharose, and total proteins were precipitated with TCA. Incorporated radioactivity was quantified by liquid scintillation counting. Results are means ± SEM for 3 observations. * denotes p < 0.05 using Student's t-test when comparing HG to LG and IBMX and DNO to C.
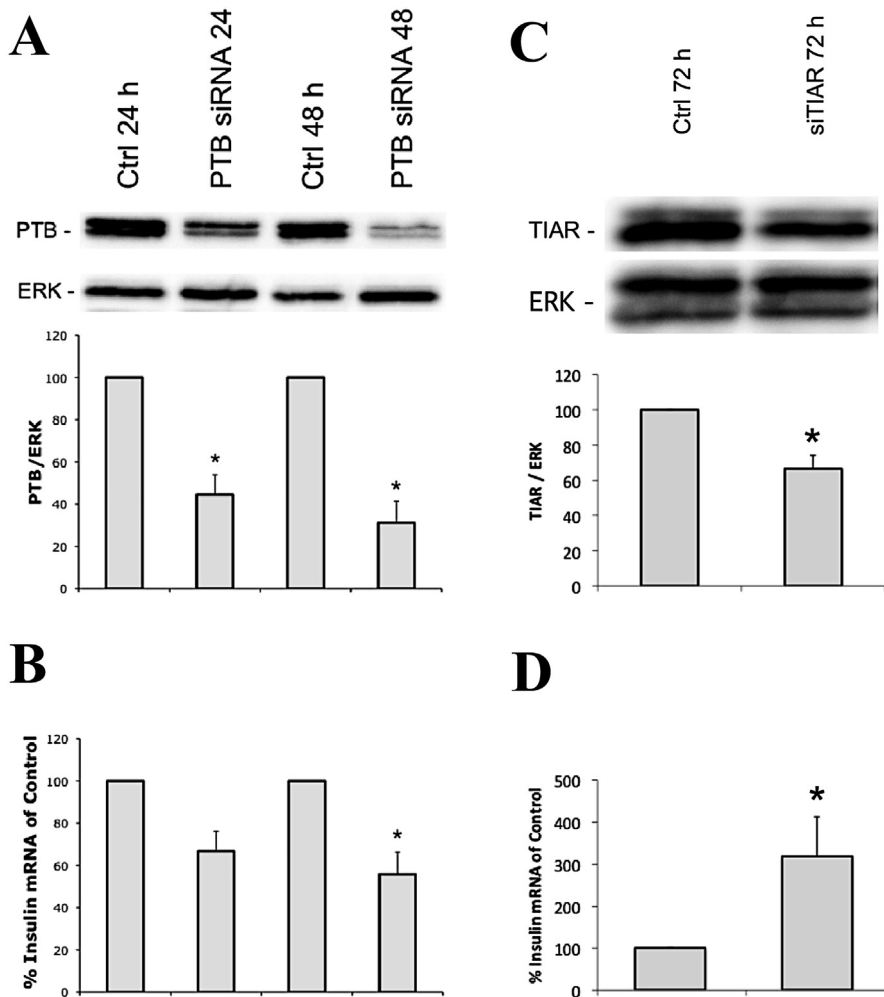
Fig. 6. Downregulation of PTB or TIAR leads to lower and higher insulin mRNA levels in INS-1 cells, respectively. INS-1 cells were transfected with either control siRNA or siRNA against PTB for 3 h using Lipofectamine according to the manufacturers recommendation. The cells where harvested after 48 h and (A) analyzed for PTB and ERK using immunoblot analysis. PTB-bands are normalized against ERK and expressed as percent of the control. A representative blot is shown in the upper panel. In (B), INS-1 cells treated as in A were analyzed using real time PCR. Insulin mRNA was normalized against beta actin and expressed as percent of control. Bars are means $\pm$ SEM for 3 observations. *Denotes $p <$ 0.05 compared to the control, using Student's paired t-test. INS-1 cells were also transfected with either control siRNA or siRNA against TIAR. The cells were harvested after 72 h and (C) analyzed for TIAR using immunoblot analysis. TIAR-bands are normalized against ERK and expressed as percent of the control. A representative blot is shown in the upper panel. In (D), INS-1 cells treated as in A were analyzed using real time PCR. Insulin mRNA was normalized against G6PDH and expressed as percent of control. Bars are means $\pm$ SEM for 3 or 4 observations. *Denotes $p < 0.05$ compared to the control, using Student's paired t-test. Full images can be found in Supplemental Fig. 6.
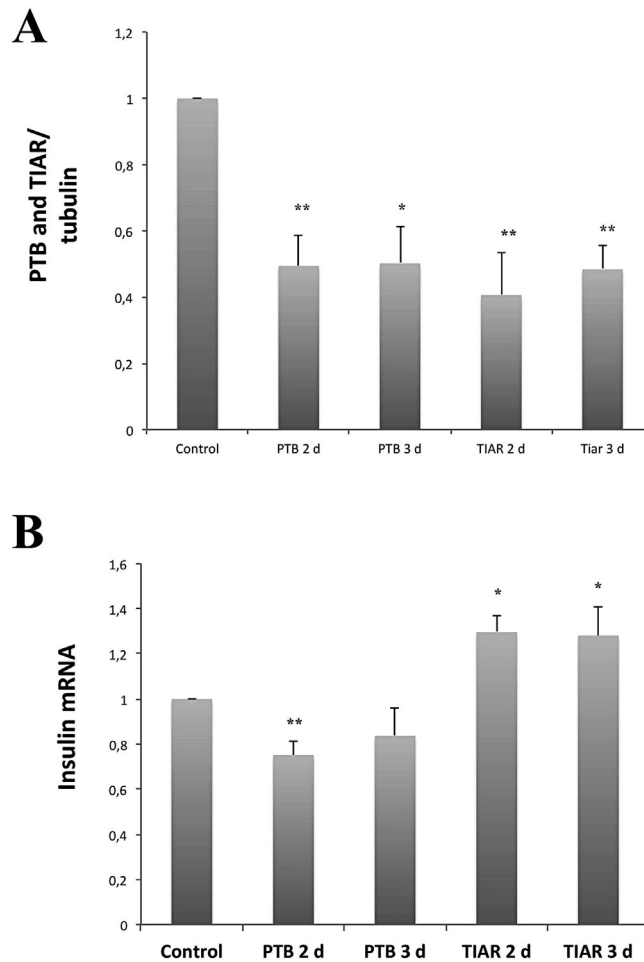
Fig. 7. Downregulation of PTB and TIAR affects insulin mRNA levels in EndoC-βH1 cells. EndoC-βH1 cells were transfected with either control siRNA or siRNA against PTB for 3 h using Dharmafect I according to the manufacturers recommendation. The cells where harvested after either 72 h and (A) analyzed for PTB and TIAR using immunoblot analysis. PTB- and TIAR-bands are normalized against tubulin and expressed relative to the control. In (B), EndoC-βH1 cells were treated as in A and analyzed using real-time PCR. Insulin mRNA was normalized against GAPDH and expressed relative to control. Bars are means ± SEM for 5–8 observations. * denotes $p < 0.05$ and ** denotes $p < 0.01$ compared to the control, using Student's paired t-test.

3.6. Thapsigargin promotes formation of TIAR-containing stress granules

Thapsigargin is a known inducer of ER stress, and in beta-cells it leads to stress granule formation and decreased insulin mRNA levels [36]. As TIAR is known to accumulate in stress granules and P-bodies [37], and to further substantiate that TIAR binding to insulin mRNA signals decreased insulin biosynthesis, we exposed INS-1 cells to thapsigargin for different time periods and analyzed stress granule formation using a TIAR antibody. As expected thapsigargin induced a pronounced and rapid increase of eIF2-alpha phosphorylation (Fig. 8A). This was paralleled by
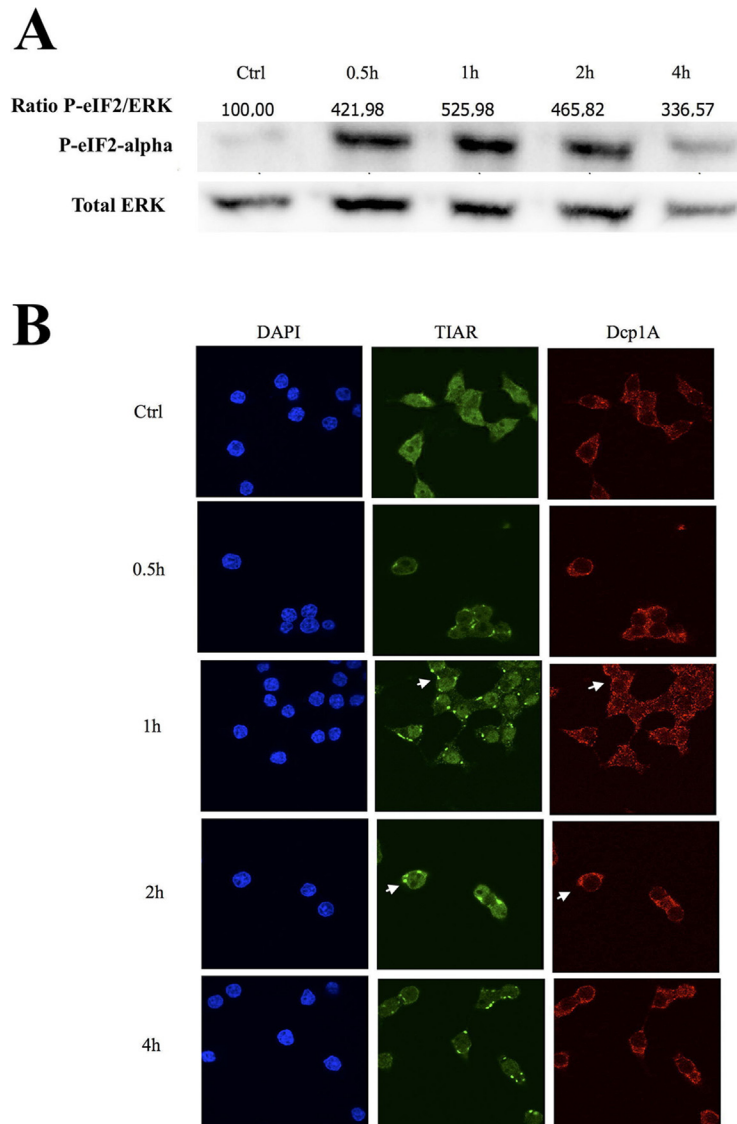
A

B

Fig. 8. Thapsigargin promotes formation of TIAR-containing stress granules in INS-1 cells. (A) Thapsigargin induced eIF2-alpha phosphorylation in INS-1 cells. Full images can be found in Supplemental Fig. 8A. (B) Immunofluorescence detection of the stress granule marker TIAR (green) and the P-body marker Dcp1A (red) in INS-1 cells after thapsigargin treatment (200 nM) for different time periods. Nuclei are visualized by DAPI (blue). Results are representative for 3 independent experiments.

the appearance of distinct, cytoplasmic stress granules, as indicated by TIAR-immunofluorescence (Fig. 8B). Thus, at conditions of ER stress, when the biosynthesis of insulin is decreased [36], TIAR redistributes from being homogenously distributed throughout the cytosol, to distinct stress granule foci. As for Dcp1a, which is a P-body marker [38], its localization appears to be in the periphery of the cells. Already at basal conditions Dcp1A is aggregated in small cytosolic foci. Thapsigargin did not increase the number of P-bodies, but the

Dcp1A intensity in P-bodies appeared stronger after thapsigargin exposure (Fig. 8B). Some P-bodies co-localized with stress granules (Fig. 8B, arrows).

4. Discussion

The rat insulin mRNA 5′- and 3′-UTRs are short, only 59 and 77 nucleotides, respectively. Yet they contain cis-elements that confer sophisticated control of messenger stability, localization and translation. Glucose augments the biosynthesis of insulin up to 10-fold [39] and insulin mRNA stability 2–3 fold [33]. These events are known to require specific sequences localized to the UTR regions [7]. Here we report that in addition to PTB, both hnRNP U and TIAR bind *in vitro* to sequences within the insulin mRNA UTRs, and that their affinity for these sequences at different conditions correlates with changes in insulin mRNA levels. More precisely, high glucose induced a modest increase in PTB and TIAR binding to the two insulin mRNA UTRs *in vitro*, which was paralleled by an enhanced insulin mRNA content. Also, down-regulation of PTB resulted in lower insulin mRNA levels. Due to the long half-life of insulin mRNA (27–72 h, [33]), we have not been able to directly quantify its stability. However, insulin mRNA is a highly abundant molecule in beta-cells [4], and it is therefore unlikely that changes in insulin mRNA levels during short-term incubations (3 h) reflect alterations in transcriptional rates. Thus, the presently observed glucose-induced increase in insulin mRNA most likely resulted from PTB-induced mRNA stabilization. This is in line with previous studies demonstrating that PTB stabilizes insulin mRNA by binding to its 3′-UTR [4, 16]. Interestingly, PTB enhances not only the stability of insulin mRNA, but also that of other mRNAs such as the messengers for CD154 [40], VEGF [41], NO synthase [42], Rab8A and B-cpx I [43].

It is not clear how a high glucose concentration promotes PTB binding to insulin mRNA. In our experiments IBMX did not increase PTB binding to the 3′- and 5′-UTR oligonucleotides, which does not give support to a role for cAMP-induced PTB phosphorylation and translocation, which was previously suggested [16]. Instead, it may be that some other glucose-induced signal promoted a post-translational modification of PTB resulting in PTB re-localization and/or altered affinity for insulin mRNA pyrimidine rich sequences. We have not been able to observe any glucose-induced shift in PTB position on SDS-PAGE, but many post-translation modifications do not result in detectable alterations in gel migration. It is also conceivable that the translation of PTB is augmented or repressed by PTB mRNA binding miRNAs. Indeed, we have recently observed that long-term glucotoxic conditions in human islets resulted in increased miR-133a levels and lower PTB protein contents [44]. MiR-133a is known to inhibit nPTB translation by binding nPTB mRNA [45], and increased miR-133a contents of human islet cells were associated with lower insulin biosynthesis rates [44]. Additional explanations may be that PTB becomes more actively recruited to the insulin

mRNA via protein-protein interactions, or that PTB binding is affected by miRNA. Indeed, it has recently been observed that miR-196b binds to the 5′-UTR of insulin mRNA thereby displacing the RNA-binding protein HuD [46], and that this leads to an increased biosynthesis of insulin. Therefore, it is possible that a short-term high glucose incubation period promotes changes in miR-levels, and that this will lead to increased PTB-insulin mRNA interaction.

It is also not clear why the binding of TIAR was modestly increased by glucose. Hypothetically, TIAR might be released from the nucleus upon glucose stimulation, as it is released from nuclei of other cells upon stress [47]. It is also possible that TIAR is modified post-translationally. Nevertheless, TIAR is in most cases associated with removal of mRNA from active translation to translational arrest in P-bodies and stress granules [37]. It may be that a high glucose concentration promotes both stimulatory and inhibitory signals, and that when the cell becomes "glucose exhausted" in response to a prolonged exposure to high glucose, the inhibitory signal predominates over the stimulatory resulting in inhibition of insulin biosynthesis.

The putative role of PTB and other RBPs in insulin biosynthesis has, to our knowledge, hitherto not yet been addressed. Our present findings point to a clear correlation between insulin mRNA stability and translation, indicating that the two processes are linked and coordinately controlled by RBP binding. Indeed, in other cell systems it is generally agreed that active translation protects against mRNA degradation and vice versa [37]. Binding of certain RBPs is generally associated with translation whereas binding of another set of RBP transfers the mRNA to a translation-inactive and degradation-active compartment [37]. The results of the present investigation are consistent with the view that PTB increases both insulin mRNA stability and translation, and that when other inhibitory RBPs bind, both processes are attenuated.

The nitric oxide donor DETAnonoate, which promotes both ER- and nitrosative stress, induced a clear increase in TIAR and hnRNP U insulin mRNA UTR binding. Furthermore, the ER stress inducer thapsigargin evoked the formation of TIAR-positive stress granules. The increased TIAR binding, and the concomitant decrease in mRNA levels and translation [36], is consistent with the view that TIAR actively participates in insulin mRNA relocalization to stress granules and p-bodies [48]. This notion is further supported by our finding that the RNAi-mediated decrease in TIAR resulted in an increased insulin mRNA level, stressing that TIAR-activity is a necessary component in suppression of insulin production. In addition, hnRNP U has been reported to participate in mRNA de-stabilization. For example, hnRNP U is known to bind the AU-rich elements (AREs) of the cyclooxytenase-2 mRNA [49]. These elements confer mRNA instability and low translational rates, and hnRNP U was demonstrated to bind AREs as a multimeric

protein complex together with HuR, TIA-1 and TIAR [49]. The exact role of hnRNP U in the metabolism of insulin mRNA remains, however, to be clarified.

In conclusion, the insulin producing cells seem to possess a control mechanism, which determines whether insulin mRNA is translated and inherently stable. At stimulatory conditions PTB binding to the mRNA ensures efficient translation and stability, but during stress, TIAR promotes mRNA removal from translation and degradation. Further studies should inform us on how the balance between TIAR and PTB is controlled, and whether diabetes in humans is associated with TIAR-induced inhibition of insulin production.

Declarations

Author contribution statement

Rikard G. Fred: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Syrina Mehrabi; Christopher M. Adams: Performed the experiments.

Nils Welsh: Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Funding statement

Additional information

Supplementary content related to this article has been published online at 10.1016/j.heliyon.2016.e00159.

Acknowledgements

References

[1] M. Cnop, N. Welsh, J.C. Jonas, A. Jorns, S. Lenzen, D.L. Eizirik, Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities, Diabetes 54 Suppl 2 (2005) S97–107.

[2] R. Tisch, H. McDevitt, Insulin-dependent diabetes mellitus, Cell 85 (1996) 291–297.

[3] B.L. Wajchenberg, beta-cell failure in diabetes and preservation by clinical treatment, Endocr. Rev. 28 (2007) 187–218.

[4] L. Tillmar, C. Carlsson, N. Welsh, Control of insulin mRNA stability in rat pancreatic islets. Regulatory role of a 3'-untranslated region pyrimidine-rich sequence, J. Biol. Chem. 277 (2002) 1099–1106.

[5] J.D. Keene, Minireview: global regulation and dynamics of ribonucleic Acid, Endocrinology 151 (2010) 1391–1397.

[6] H. Hieronymus, P.A. Silver, A systems view of mRNP biology, Genes Dev. 18 (2004) 2845–2860.

[7] B. Wicksteed, T.P. Herbert, C. Alarcon, M.K. Lingohr, L.G. Moss, C.J. Rhodes, Cooperativity between the preproinsulin mRNA untranslated regions is necessary for glucose-stimulated translation, J. Biol. Chem. 276 (2001) 22553–22558.

[8] B. Wicksteed, Y. Uchizono, C. Alarcon, J.F. McCuaig, A. Shalev, C.J. Rhodes, A cis-element in the 5' untranslated region of the preproinsulin mRNA (ppIGE) is required for glucose regulation of proinsulin translation, Cell Metab. 5 (2007) 221–227.

[9] E.J. Wagner, M.A. Garcia-Blanco, Polypyrimidine tract binding protein antagonizes exon definition, Mol. Cell Biol. 21 (2001) 3281–3288.

[10] A. Moreira, Y. Takagaki, S. Brackenridge, M. Wollerton, J.M. Manley, N.J. Proudfoot, The upstream sequence element of the C2 complement poly(A) signal activates mRNA 3' end formation by two distinct mechanisms, Genes Dev. 12 (1998) 2522–2534.

[11] C.A. Cote, D. Gautreau, J.M. Denegre, T.L. Kress, N.A. Terry, K.L. Mowry, A Xenopus protein related to hnRNP I has a role in cytoplasmic RNA localization, Mol. Cell 4 (1999) 431–437.

[12] S.A. Mitchell, K.A. Spriggs, M. Bushell, J.R. Evans, M. Stoneley, J.P. Le Quesne, R.V. Spriggs, A.E. Willis, Identification of a motif that mediates polypyrimidine tract-binding protein-dependent internal ribosome entry, Genes Dev. 19 (2005) 1556–1571.

[13] R.G. Fred, N. Welsh, Increased expression of polypyrimidine tract binding protein results in higher insulin mRNA levels, Biochem. Biophys. Res. Commun. 328 (2005) 38–42.

[14] R.G. Fred, M. Sandberg, J. Pelletier, N. Welsh, The human insulin mRNA is partly translated via a cap- and eIF4A-independent mechanism, Biochem. Biophys. Res. Commun. 412 (2011) 693–698.

[15] K.P. Knoch, S. Nath-Sain, A. Petzold, H. Schneider, M. Beck, C. Wegbrod, A. Sönmez, C. Münster, A. Friedrich, M. Roivainen, M. Solimena, PTBP1 is required for glucose-stimulated cap-independent translation of insulin granule proteins and Coxsackieviruses in beta cells, Mol. Metab. 3 (2014) 518–530.

[16] K.P. Knoch, H. Bergert, B. Borgonovo, H.D. Saeger, A. Altkruger, P. Verkade, M. Solimena, Polypyrimidine tract-binding protein promotes insulin secretory granule biogenesis, Nat. Cell Biol. 6 (2004) 207–214.

[17] D.J. Hogan, D.P. Riordan, A.P. Gerber, D. Herschlag, P.O. Brown, Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system, PLoS Biol. 6 (2008) e255.

[18] H. Li, W. Chen, Y. Zhou, P. Abidi, O. Sharpe, W.H. Robinson, F.B. Kraemer, J. Liu, Identification of mRNA binding proteins that regulate the stability of LDL receptor mRNA through AU-rich elements, J. Lipid Res. 50 (2009) 820–831.

[19] P. Ravassard, Y. Hazhouz, S. Pechberty, E. Bricout-Neveu, M. Armanet, P. Czernichow, R. Scharfman, A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion, J. Clin. Invest. 121 (2011) 3589–3597.

[20] R.A. Zubarev, D.M. Horn, E.K. Fridriksson, N.L. Kelleher, N.A. Kruger, M. A. Lewis, B.K. Carpenter, F.W. McLafferty, Electron capture dissociation for structural characterization of multiply charged protein cations, Anal. Chem. 72 (2000) 563–573.

[21] M.M. Savitski, F. Kjeldsen, M.L. Nielsen, R.A. Zubarev, Complementary sequence preferences of electron-capture dissociation and vibrational excitation in fragmentation of polypeptide polycations, Angew. Chem. Int. Ed. Engl. 45 (2006) 5301–5303.

[22] M.L. Nielsen, M.M. Savitski, R.A. Zubarev, Improving protein identification using complementary fragmentation techniques in fourier transform mass spectrometry, Mol. Cell. Proteomics. 4 (2005) 835–845.

[23] S.D. Kulkarni, B. Muralidharan, A.C. Panda, B. Bakthavachalu, A. Vindu, V. Seshadri, Glucose-stimulated translation regulation of insulin by the 5' UTR-binding proteins, J. Biol. Chem. 286 (2011) 14146–14156.

[24] E.K. Lee, W. Kim, K. Tominaga, J.L. Martindale, X. Yang, S.S. Subaran, O. D. Carlson, E.M. Mercken, R.N. Kulkarni, W. Akamatsu, H. Okano, N.I. Perrone-Bizzozero, R. de Cabo, J.M. Egan, M. Gorospe, RNA-binding protein HuD controls insulin translation, Mol. Cell 45 (2012) 826–835.

[25] K.F. Malik, H. Jaffe, J. Brady, W.S. Young 3rd, The class III POU factor Brn-4 interacts with other class III POU factors and the heterogeneous nuclear ribonucleoprotein U, Brain Res. Mol. Brain Res. 45 (1997) 99–107.

[26] F. Gohring, F.O. Fackelmayer, The scaffold/matrix attachment region binding protein hnRNP-U (SAF-A) is directly bound to chromosomal DNA in vivo: a chemical cross-linking study, Biochemistry 36 (1997) 8276–8283.

[27] M. Kiledjian, G. Dreyfuss, Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box, EMBO J. 11 (1992) 2655–2664.

[28] J. Hui, L.H. Hung, M. Heiner, S. Schreiner, N. Neumuller, G. Reither, S.A. Haas, A. Bindereif, Intronic CA-repeat and CA-rich elements: a new class of regulators of mammalian alternative splicing, EMBO J. 24 (2005) 1988--1998.

[29] B. Hahm, Y.K. Kim, J.H. Kim, T.Y. Kim, S.K. Jang, Heterogeneous nuclear ribonucleoprotein L interacts with the 3' border of the internal ribosomal entry site of hepatitis C virus, J. Virol. 72 (1998) 8782–8788.

[30] B.J. Hamilton, X.W. Wang, J. Collins, D. Bloch, A. Bergeron, B. Henry, B. M. Terry, M. Zan, A.J. Mouland, W.F. Rigby, Separate cis-trans pathways post-transcriptionally regulate murine CD154 (CD40 ligand) expression: a novel function for CA repeats in the 3'-untranslated region, J. Biol. Chem. 283 (2008) 25606–25616.

[31] J.R. Buchan, R. Parker, Eukaryotic stress granules: the ins and outs of translation, Mol. Cell 36 (2009) 932–941.

[32] J.L. Taupin, Q. Tian, N. Kedersha, M. Robertson, P. Anderson, The RNA-binding protein TIAR is translocated from the nucleus to the cytoplasm during Fas-mediated apoptotic cell death, Proc. Natl. Acad. Sci. U S A 92 (1995) 1629–1633.

[33] M. Welsh, D.A. Nielsen, A.J. MacKrell, D.F. Steiner, Control of insulin gene expression in pancreatic beta-cells and in an insulin-producing cell line, RIN-

5F cells. II. Regulation of insulin mRNA stability, J. Biol. Chem. 260 (1985) 13590–13594.

[34] M. Welsh, N. Scherberg, R. Gilmore, D.F. Steiner, Translational control of insulin biosynthesi. Evidence for regulation of elongation, initiation and signal-recognition-particle-mediated translational arrest by glucose, Biochem. J. 235 (1986) 459–467.

[35] D.L. Eizirik, M.I. Darville, beta-cell apoptosis and defense mechanisms: lessons from type 1 diabetes, Diabetes 50 Suppl 1 (2001) S64–69.

[36] Y.Y. Jang, N.K. Kim, M.K. Kim, H.Y. Lee, S.J. Kim, H.S. Kim, H.Y. Seo, I.K. Lee, K.G. Park, The Effect of Tribbles-Related Protein 3 on ER Stress-Suppressed Insulin Gene Expression in INS-1Cells, Korean Diabetes J. 34 (2010) 312–319.

[37] N. Cougot, S. Babajko, B. Séraphin, Cytoplasmic foci are sites of mRNA decay in human cells, J. Cell Biol. 165 (2004) 31–40.

[38] D.F. Steiner, S.J. Chan, M.J. Welsh, S.C. Kwok, Structure and evolution of the insulin gene, Annu. Rev. Genet. 19 (1985) 463–484.

[39] B.J. Hamilton, A. Genin, R.Q. Cron, W.F. Rigby, Delineation of a novel pathway that regulates CD154 (CD40 ligand) expression, Mol. Cell. Biol. 23 (2003) 510–525.

[40] L.S. Coles, M.A. Bartley, A. Bert, J. Hunter, S. Polyak, P. Diamond, M.A. Vadas, G.J. Goodall, A multi-protein complex containing cold shock domain (Y-box) and polypyrimidine tract binding proteins forms on the vascular endothelial growth factor mRNA. Potential role in mRNA stabilization, Eur. J. Biochem. 271 (2004) 648–660.

[41] M. Soderberg, F. Raffalli-Mathieu, M.A. Lang, Inflammation modulates the interaction of heterogeneous nuclear ribonucleoprotein (hnRNP) I/polypyrimidine tract binding protein and hnRNP L with the 3'untranslated region of the murine inducible nitric-oxide synthase mRNA, Mol. Pharmacol. 62 (2002) 423–431.

[42] J.F. Porter, S. Vavassori, L.R. Covey, A polypyrimidine tract-binding protein-dependent pathway of mRNA stability initiates with CpG activation of primary B cells, J. Immunol. 181 (2008) 3336–3345.

[43] R.G. Fred, C.H. Bang-Berthelsen, T. Mandrup-Poulsen, L.G. Grunnet, N. Welsh, High glucose suppresses human islet insulin biosynthesis by inducing miR-133a leading to decreased polypyrimidine tract binding protein-expression, PLoS ONE 5 (2010) e10843.

[44] P.L. Boutz, G. Chawla, P. Stoilov, D.L. Black, MicroRNAs regulate the expression of the alternative splicing factor nPTB during muscle development, Genes Dev. 21 (2007) 71–84.

[45] A.C. Panda, I. Sahu, S.D. Kulkami, J.L. Martindale, K. Abdelmohsen, A. Vindu, J. Joseph, M. Gorospe, V. Seshadri, miR-196b-mediated translation regulation of mouse insulin2 via the 5′UTR, PLoS One 9 (2014) e101084.

[46] A. Esclatine, B. Taddeo, B. Roizman, Herpes simplex virus 1 induces cytoplasmic accumulation of TIA-1/TIAR and both synthesis and cytoplasmic accumulation of tristetraprolin, two cellular proteins that bind and destabilize AU-rich RNAs, J. Virol. 78 (2004) 8582–8592.

[47] R. Parker, U. Sheth, P bodies and the control of mRNA translation and degradation, Mol. Cell 25 (2007) 635–646.

[48] N. Kedersha, P. Anderson, Mammalian stress granules and processing bodies, Methods Enzymol. 431 (2007) 61–81.

[49] S.J. Cok, S.J. Acton, A.R. Morrison, The proximal region of the 3'-untranslated region of cyclooxygenase-2 is recognized by a multimeric protein complex containing HuR, TIA-1, TIAR, and the heterogeneous nuclear ribonucleoprotein U, J. Biol. Chem. 278 (2003) 36157–36162.