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Long-RNA sequencing and ribosome profiling of inflamed beta-cells reveal an extensive translome landscape

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ABSTRACT

Type 1 diabetes is an autoimmune disease characterized by autoreactive T-cell mediated destruction of the insulin-producing pancreatic beta-cells. Increasing evidence suggest that the beta-cells themselves contribute to their own destruction by generating neo-antigens through the production of aberrant or modified proteins that escape central tolerance. We have recently demonstrated that ribosomal infidelity amplified by stress could lead to the generation of neoantigens in human beta-cells, emphasizing the participation of nonconventional translation events to autoimmunity, as occurring in cancer or virus-infected tissues. Using a transcriptome-wide profiling approach to map translation initiation start sites in human beta-cells under standard and inflammatory conditions, we identify a completely new set of polypeptides derived from non-canonical start sites and translation initiation within lncRNA. Our data underline the extreme diversity of the beta-cell translome and may reveal new functional biomarkers for beta-cell distress, disease prediction and progression and therapeutic intervention in type 1 diabetes.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the selective and progressive destruction of the insulin-producing pancreatic beta-cells by the invading immune cells(1-3) . T-cells of high affinity are normally eliminated in the thymus by negative selection in order to establish central tolerance. In autoimmune disease, impaired thymic education or low affinity T-cells are believed to be responsible for the immune attack directed against native self-proteins(4). Yet, there is increasing evidence that local inflammation or other forms of stress combined with genetic predisposition leads to the generation and accumulation of aberrant or modified proteins to which central tolerance is lacking and increased visibility of beta-cells to immune cells (5-7). Metabolic and inflammatory stress may perturb the cellular equilibrium and affect high fidelity transcriptional and translational processes during conversion of the genetic information into proteins. We and others have shown increased RNA splicing in beta-cells when maintained under pathophysiological conditions(8; 9), as well as T-cell autoreactivity against deamidated autoantigens and fusion epitopes illustrating that transcriptional, post-transcriptional and post-translational processes can be affected during insulinitis, supporting the participation of unconventional antigens in T1D pathology(10-13). While transcription regulation is a slow process, translation and ribosome position along RNA molecules can change upon stress in a matter of seconds (14). Many genes involved in the unfolded protein response to stress are regulated at a translational level. This swift cell adaptation is illustrated by the regulation of stress responsive genes (i.e. *ATF4*, *ATF5*, *GADD34* or *CHOP*), where the activation of the PERK sensor within the ER triggers phosphorylation of the translation initiation factor eIF2 and the translational activation of ATF4 controlling the unfolded protein response(15). In this classical model of translation initiation, the RNA precursor is converted into mature RNA by addition of 7-

methyguanosine (m7G) at its 5'; addition of a poly A tail in 3' end; and splicing of intronic regions. Canonically, 5' capping of the mRNA and binding of the 40s ribosomal subunit via the eIF4 factor represents the earliest stage of translation initiation from which mRNA scanning process continues until recognition of a start codon where the complete 80s ribosome complex starts translation upon recognition of an AUG by the first methionine charged initiator tRNA. Based on this model, the identification of proteins expressed by a cell can be predicted from transcriptomics data analysis. However, the translation process is not flawless and non-canonical initiation can occur(16). In addition, environmental perturbation may affect translation fidelity and cellular stress aggravates translation initiation at alternative start codons with possible consequences at the immunological level(17). In fact, changes in reading frame will necessarily affect the amino acid sequence encoded by RNA molecules. Being recognized as danger signal or just as a translational error, these so-called 'alternative open reading frames products may trigger cell destruction by the immune surveillance. These non-conventional translational events arising from translation of normally untranslated regions (UTR), ribosomal frame-shifting or alternative translation initiation (uTIS or dTIS), represent a unique pool of antigenic peptides that rapidly undergo degradation to the proteasome(18; 19). Such epitopes have been described frequently in tumor cells (i.e. ARFs, MELOE, NY-ESO-1, and LAGE-1), and are currently explored as tumor biomarkers or as therapeutic targets to promote a specific anti-tumor response(13). Surprisingly, in the context of autoimmunity, these neoepitopes have rarely been investigated(20), in spite of reports estimating that up to 70% of peptides presented at the cell surface in MHC class I may be derived from defective ribosomal products(21). We have recently shown that translation of the human insulin mRNA can generate translational errors that participate to beta-cell destruction providing the first evidence for the implication of translational "junk" products in triggering

autoimmunity(22). However, current transcriptomic, proteomic and peptidomic approaches aiming at extending our view on the beta-cell ligandome and improving our understanding on potential involvement of beta-cell to T1D pathogenesis(23) are not compatible with the detection of out-of-frame rapidly degraded polypeptide products, thereby masking important features of the beta-cell ligandome.

We used a novel unbiased proteogenomic approach to identify all possible open reading frames (ORFs) generated by beta-cells maintained in normal and T1D pathophysiological conditions. Our results uncover evidence for translation initiation in novel RNA transcripts as well as long non-coding RNA. Moreover, we show that cytokine treatment leads to profound changes in genes involved in MHC-class I processing and a significantly increased number of ORFs per transcript and in particular an increased ribosome density within 5'-UTR regions. Altogether, our data present new insights in the dynamic regulation of transcription and translation revealing an extended beta-cell translome.

RESEARCH DESIGN AND METHODS

Cells and reagents.

EndoC- β H1 cells, kindly provided by Dr. Raphael Scharfmann (Paris Descartes University, France)(24) , were maintained in low glucose DMEM supplemented with 5.5 μ g/ml human transferrin, 10 mM Nicotinamide, 6.7 ng/ml Selenit, 50 μ M β -mercaptoethanol, 2% human albumin, 100 units/ml Penicillin and 100 μ g/ml streptomycin. Cells were seeded in ECM, fibronectin pre-coated culture plates. Inflammatory stress was induced by a mixture of 1000 U/ml IFN γ and 2 ng/ml IL1 β for 24 h. Upon incubation, cells were treated with 2ug/ml harringtonine for 30 minutes at 37°C, as previously described(16). Cycloheximide (100ug/ml) was added for 30 seconds. Ten million cells were used per condition. Subsequent cell lysis was performed without freezing as described using 900 ul of lysis buffer. The clarified lysates were stored overnight at -80 °C.

Nuclease footprinting and ribosome recovery.

The materials and methods used are adapted from previously described protocol(25). Following cell lysis (20mM Tris Cl (Ph 7.4), 150mM NaCl, 5mM MgCl₂, 1mM DTT supplemented with 100ug/ml cycloheximide, 1% Triton X-100 and 25U/ml Turbo DNaseI), ribosome complexes were purified by adding three times the volume of sucrose cushion to the clarified lysate solution in Thickwall Polycarbonate Tube, 13x64mm (Beckman Coulter Life Sciences, part no. 344645). Ribosomes were pelleted by centrifugation using Delrin Tube Adapters (Beckman Coulter Life Sciences, part no. 303313) in a Type 70.1 Ti Fixed-Angle Titanium Rotor (Beckman Coulter Life Sciences, part no. 337922). The ribosomal pellets were further lysed using 300 μ l Buffer ML from the Nucleospin miRNA Kit (Bioke, product code 740971). Lysates were stored overnight at -80

°C and RNA purified using Nucleospin miRNA according to the manufacturer's guidelines. The RNA was precipitated using Glycogen, RNA grade (ThermoFisher Scientific, cat. no. R0551) and resuspended in 10 µl of DEPC-Treated Water (ThermoFisher Scientific, cat. no. AM9920).

Footprint fragment purification.

Purified RNA was denaturated (98%(vol/vol) formamide, 10mM EDTA, 300ug/ml bromophenol blue) and load on 18 % (wt/vol) polyacrylamide TBE-urea gel (200 V for 90 min in 1x TBE buffer). Molecular weight ladder a mix was prepared of 5 µl microRNA Marker (New England Biolabs, cat. no. N2102), and 0.5 µl Small RNA Marker (Abnova, cat. no. R0007). The SYBR Gold (ThermoFisher Scientific, cat. no. S11494) stained gel was visualised using a ChemiDoc MP Imaging System (Bio-Rad). RNA was extracted from excised gel slices spanning the 26 nt to 34 nt region, using the ZR small-RNA PAGE Recovery Kit (Zymo Research, cat. no. R1070) according to the manufacturer's protocol. The size-selected RNA was stored at -80 °C.

Dephosphorylation and linker ligation.

Dephosphorylation reaction and linker ligation were performed using the Universal miRNA Cloning linker (New England Biolabs, cat. no. S1315S) in a T100 Thermal Cycler (Bio-Rad, product number 1861096). RNA was precipitated using Glycogen, RNA grade (ThermoFisher Scientific, cat. no. R0551) and resuspended in 10 µl of DEPC-treated Water (ThermoFisher Scientific, cat. no. AM9920). Size separation and gel extraction of the ligation reaction were performed as described in the footprint fragment purification section.

Reverse transcription and circularization.

The reverse transcription reaction was performed in a T100 Thermal Cycler using RT primers). Gel extraction were performed as described in the footprint fragment purification section and cDNA circularized using CircLigase following guidelines from the manufacturer.

Ribosomal RNA depletion and barcoding.

The rRNA depletion was performed using a T100 thermal cycler (Bio-Rad) and a Thermomixer 5350 Mixer (Eppendorf) using depletion primers (**Table 1**). PCR was performed using library preparation primers. PCR strips were removed at the end of the extension step after 7, 8, 9, 10, 11, and 12 cycles. The samples were separated by electrophoresis using a 9 % (wt/vol) polyacrylamide TBE-urea gel at 180 V for 45 min in 1x TBE. As molecular weight marker 5 µl 50 bp DNA Ladder (New England Biolabs, cat. no. B7025) was included. The SYBR gold stained gel was visualized using a ChemiDoc MP (Biorad). Excised gel slices containing the amplicons were crushed and soaked overnight at room temperature under gentle mixing, using 400 µl diffusion buffer for PAGE gels (500 mM ammonium, pH 8.0, 0.1 % SDS, 1 mM EDTA, 10 mM magnesium acetate) and Nucleospin Gel & PCR Clean-up (Bioke, product code 740609). Subsequently, the libraries were isolated from the extraction mix according to the manufacturer's recommendations and eluted in 30 µl Elution Buffer NE. The libraries were quantified and characterized using the Experion DNA 1K Analysis Kit (Bio-Rad, product number 7007107) on a Experion Automated Electrophoresis System (Bio-Rad) following the manufacturer's protocol.

RESULTS

Precise mapping of the translation initiation start sites in beta-cells by ribosome profiling.

To evaluate translational changes induced by proinflammatory cytokines during the early events of type 1 diabetes, we treated the human beta-cell line EndoC- β H1 with proinflammatory cytokines (IFN γ and IL1 β) and isolated ribosome protected fragments to map translation initiation sites (TIS) (n=3 independent experiments). Using a combined treatment with harringtonine that incorporates at the A-site of the large 60S ribosomal subunit, thereby blocking the entry of a second tRNA, and cycloheximide that displays a high affinity for the uncharged tRNA E-pocket of the 80S complex thereby preventing further elongation, we froze ribosomes at the initiation of translation(16; 25; 26). Following RNaseI treatment, polysomes were purified and ribosomes protected fragments (RPFs) isolated to generate ribosome footprint libraries containing the translation initiation sites(25) (**Fig.1A**). As anticipated from the stoichiometric hindrance of ribosomes on RNA molecules, the length of fragments sequenced ranged from 26 to 31 nucleotides independently of the treatment (**Fig.1B**). Alignment of the RPFs to the human transcriptome revealed an accumulation of reads at the beginning of the translated region of the transcripts, consequence of the high ribosome occupancy at the translation start (**Fig.S1A**). For reads of same length, P-site offsets were computed to precisely map the position of the ribosome P-site, corresponding to the start codon, on the RNA footprint(27). Shorter incubation with harringtonine failed to stop the ribosome complex at the start codon leading to noise during elongation and accumulation of reads at 3' UTR resulting from ribosome stalling before completing peptide synthesis (**Fig.S1B**). The occupancy profiles of the RPFs 5' and 3' end extremities around the referenced start codons, showed accumulation of read ends at locations +/- 12 (**Fig.S1C**), validating both ribosomal freezing and computational calculation allowing mapping of the start

site (**Fig.S1D-E**). In order to eliminate false-positives and minimize background artifacts resulting from the speed of transcript translation, incomplete blockade by translation inhibitors or possible non-ribosomal protein complexes interacting with the mRNA, we developed an algorithm based on the read distribution surrounding the suspected P-site to identify the most relevant translation initiation starts. Reasoning that the accumulation of RPFs at a start position are normally distributed, the read spread in flanking position is determined by the number of reads of the peak, we defined an enrichment method using a dynamic sliding window encompassing each P-site site position (**Supplementary materials and Fig.S2A-B**). Significance testing was performed using a negative binomial regression model, and samples belonging to different library preparations and treatment were statistically paired. Using this enrichment method, we identified 15014 start sites within 5529 genes in both untreated and cytokine treated samples (**Fig.1C and data file S1**). Codon occupancy analysis at the E, P and A pockets indicated a conserved AUG motif at the P-site within a Kozak consensus sequence both in non-treated and cytokine- treated samples (**Fig.1D**).

Direct RNA sequencing and Ribo-seq technology reveals active translation in novel transcript variants and long non-coding RNA.

In order to evaluate the impact of cytokine stimulation on translation, we performed a negative binomial regression on the read numbers at the P-site position. As expected, pathways related to inflammatory response, such as antigen processing, presentation and apoptosis, were induced upon treatment (**Fig.2A**). More specifically, translation initiation within transcripts of these inflammatory pathways was significantly upregulated (p-value < 0.05 and log₂ Fold Change >1.5) (**Fig.2B**). Increased expression of key proteins for secretory pathway (CHGA), IFN signaling

(STAT1, STAT2) and antigen processing (PSMB8, PSMB9 and PSMB10) was validated by western blot in EndoC- β H1 cells and confirmed in primary human islets (Supplementary **Fig. S3**). While these data do not provide proofs for expression of the alternative ORFs, they contribute to technical validation of the ribosome profiling approach. Despite a stringent detection method, 17% of P-sites identified corresponded to referenced annotated start codon (**Fig.2C**). Importantly, we identified several misannotated translational start sites (i.e. TAP1 and PSMD8) experimentally validated in previous ribosome profiling assays (TISdb database)(28) or extra start sites previously confirmed by mass spectrometry (i.e. YBX1 and PGRMC1) (**Fig.S4A-D**). By reconstituting the 3 base periodicity move of the ribosomes on RNA molecule from the identified start sites, our analysis revealed a wide range of novel or modified ORFs leading to translation rearrangements (upstream initiation: uORF that ends before the coding sequence (CDS), N-terminal extension of annotated protein, overlapping uORF spanning 5'-UTRs and CDS on an alternative frame/ translation frameshift: internal off-frame, downstream initiation: initiation on the 3'-UTR) (**Fig.2D**). Moreover, we detected a considerable fraction (7%) of the RPFs mapped to non-coding RNA and preferentially in long non-coding RNA (**Fig.2E-F**). The polypeptide lengths of ORFs initiated at alternative start sites in coding (n=25466 in non-treated and n= 25984 in cytokine condition) or non-coding RNA (n=2634 in non-treated and n= 2381 in cytokine) were smaller than canonical ORFs (**Fig.2G**). These differences in peptide sizes observed are in line with previous studies conducted on embryonic stem cells and cancer cell lines(29; 30).

Moreover, nearly 20% of the purified ribosome footprints did not align to the referenced transcriptome indicating the presence of TIS on novel transcripts (**Fig.3A and data file S2**). In order to further explore the beta-cell transcriptome and translome, we stimulated EndoC- β H1 cells with cytokines and performed direct long RNA sequencing in search for novel transcript

variants (**Supplementary materials and Fig.3B**). Following a differential expression analysis, we identified a classical transcriptional inflammatory signature on T1D susceptible genes(31; 32) (**Fig.3C and data file S3**). Long RNA sequencing identified 6892 new transcripts, among which 37 were specifically present under inflammatory condition (**Fig.3D**). In order to validate our results, we aligned reads from our *denovo* transcriptome to RNA sequencing datasets from pancreatic β cells (33). As described in Supplementary **Figure S5A-B** and **data file S4**, 74% of the newly identified transcripts spanning over splicing junctions in beta cell line (77% of the T1D susceptible genes) are supported by RNA-seq reads from primary beta cell enriched fraction. In addition, we evaluated the expression pattern of 2 novel cytokines specific transcripts (i.e. PSMB9 TALONT000709680 and HLA-C TALONT000700063) in EndoCBH1 and primary human islets. As shown in Figure S5, classical RT-PCR analysis confirmed the cytokine specificity in EndoC- β H1, the expression in islets may illustrate the difference between EndoC- β H1 and primary beta cell but more likely reflect the presence of other islet cell types in the RNA preparation) (**Fig. S5C-F**).

Alignment of the RPFs to the newly defined beta-cell transcriptome revealed over 1745 TIS located on the novel transcripts, including 499 TIS specifically detected upon treatment (**Fig.3E**). Importantly, these TIS were surrounded by Kozak-like sequences and could potentially give rise to ORFs ~150aa long (**Fig.3F-G and data file S5**). By integrating transcriptome and translome datasets, we evaluated the transcriptional and translational adaptive response to inflammatory cytokines in beta-cells. CST3, PRPS1 and MRPS31 in particular appear regulated at the translational level rather than transcriptional level (**Fig.3H and data file S6**).

Cytokine treatment distorts translation fidelity triggering generation of alternative translation initiation sites.

Metagene distribution of the TIS throughout the whole transcriptome, indicated that cytokine stimulation led to a significant decrease in the number of transcripts presenting a unique start site ($P\text{-value} < 0.0001$), and an increased proportion of transcripts with multiple TIS ($P\text{-value} < 0.0001$) suggesting that inflammation may actively trigger the generation of intra-transcript initiation events (**Fig.4A**). Moreover, we observed an increased number of P-sites within 5'-UTR regions (~4% increase $P\text{-value} < 0.0001$) in cytokine condition when compared to non-treated samples which is in line with studies showing a slower progression of the ribosome complex on RNA during stress condition(34; 35) (**Fig.4B**). These changes were associated with a modification of the initiation start codon motif (CUG) (**Fig.4C**). Differential read expression at the alternative TIS showed increased translation efficiency within transcripts involved in the inflammation response, in particular in the 5'UTR and CDS region of HLA, TAP1, TAP2, IFIH1 (**Fig.4D**). A similar inflammatory response was obtained within newly identified HLA-A and HLA-E transcripts and within transcripts encoding for immunoproteasome subunits (e.g. PSMB9) (**Fig.S6A**). Within non-coding RNA a higher translation initiation was also detected in non-coding isoforms of PSMB9 and ARF4 transcripts (**Fig.S6B**). A differential ribosomal occupancy was found specifically in long-non coding RNA in particular within EBLN3P, AL627171.4 or AC112487.1 suggesting an increased production of those small ORF during inflammation (**data file S7**).

Combining information on translation efficiency at different start sites, we observed a poor correlation between differential read expression at the alternative P-site location and changes at their canonical counterparts ($R=0.32$), suggesting specific regulatory mechanisms for translation initiation at each position. Cytokine treatment prevented or favored ribosome docking to

alternative positions (n=189 and n=135, respectively) (**Fig.4E and data file S8**). Pathway analysis performed on these transcripts pointed to an active regulatory mechanism driven by inflammatory signals to adjust RNA splicing and translation machinery in response to stress (**Fig.S7**). Alternative translation initiation within MAGED2, VEGFA and CHGA transcripts was induced by cytokine stimulation. While no polypeptide could be expected from MAGED2 alternative start (AAT_{Start}TGA_{Stop}), we observed an increased ribosome density during inflammation at alternative position within the 5'-UTR and 3'-UTR region of the CHGA transcript that could generate short polypeptides. Using a GFP fusion construct, in which the GFP ORF is positioned in frame with the CGG-103; CCG-40 and CTG-22 start codons identified by ribosome profiling, we validated that the 5'-UTR region can be used to initiate protein translation in a +2 frame (**Fig.S8**). Of note, translation initiation within 3'-UTR at +1512 could generate a potentially immunogenic polypeptide carrying a strong HLA-A2 epitope. Similarly, within VEGFA transcripts, a novel upstream TIS was identified located upstream the canonical CUG (VEGFA-111), upregulated upon treatment, that can potentially generate two strong HLA-A2 binders (KVSDLLLGV and LLLGVTAGA) (**Fig.4E-F**). Of note, we detected an extra in frame start site, in position +3 (data not shown), in addition to the canonical CUG start codon (position 0) that carries the SRFGGAVVR epitope, ~7-fold upregulated upon cytokine treatment, which could drive increased presentation of previously identified cryptic VEGFA epitope(36).

Regarding insulin gene products, a high ribosome density was detected at the canonical start site at position 0, as expected. Also, extra alternative starts were detected within the 5'-UTR region at position -44; -34 generating overlapping ORFs (upstream ORF in +1 and +2 reading frame respectively) (**Fig.5A and data file S9**). Moreover, we detected an accumulation of ribosomes downstream the canonical start site at position 286, confirming that this AUG may serve as docking

site for translation initiation of the immunogenic out of frame INS-DRiP polypeptide we described earlier(22). Using long RNA sequencing, 5 novel INS transcripts were identified resulting from size variations within exon 1 and 3, intron 1 retention, or 3' extension. While TALONT000499163, TALONT000499165, TALONT000499178 and TALONT000499193 were in low abundance, the INS-IGF2 transcript (TALONT000499153), deriving from the fusion between exon 3 of the INS gene and the INS-IGF2 intronic region is the third most abundant INS transcript expressed by beta cells, representing 3% of the total INS reads. Using a primer set spanning the end of the classical INS transcript, located in INS exon 3 and in the INS-IGF2 intergenic region, we validated the presence of the TALONT000499153 transcript in EndoC- β H1 cells and primary islets and validated the similar transcript abundance, measured by longRNA sequencing, in normal and cytokine condition (**Fig.5B**). Ribosome density on this region identified a potent translation initiation site that would lead to the generation of a 116 amino acid polypeptide carrying the insulin DRIP epitope and bearing another potential HLA-A2 binder (**Fig.5C**). To validate alternative translation initiation within INS transcripts in EndoC- β H1 cells, we tested the MLYQHLLPL epitope presentation to DRiP specific CD8 T-cell clone. Using JY cells pulsed with insulin derived peptides (PPI: ALWGPDPA AAA; B-chain: HLVEALYLV ; DRiP: MLYQHLLPL), we confirmed DRiP T-cell specificity by detecting T cell activation only in presence of the cognate peptide (**Fig. 5D**). Following genetic modification to express HLA-A2 in EndoCBH1 cells, we validated that the exogenous HLA-A2 expression in presence or absence of cytokines was similar to the endogenous HLA class I expression (**Fig. 5E**). After co-culture with the DRiP specific CD8 T cells, we detected an increase CD8 activation, measured by CD107a staining and MIP1 β release in presence of cytokine stimulation (**Fig. 5F**) confirming the importance of an HLA

hyperexpression in beta cell destruction process but also providing proof for processing and presentation of the N-term fragment of the INS alternative ORF in EndoC- β H1 cells.

DISCUSSION

The Defective Ribosomal Protein (DRIP) theory arose in 1996(21) when discrepancies between the ligandome and proteome were found in virally infected cells, as a plausible explanation for T cell reactivity against flu derived peptides detected prior synthesis of their source proteins. Originally identified as prematurely terminated polypeptides and misfolded polypeptides produced from translation of bona-fide mRNAs in the proper reading frame(21), DRiPs include all alternative translation initiation products, including UTR translation and ribosomal frame-shifts products, each of these being susceptible to generate a new pool of antigenic peptides that would not share any, or little, amino acid similarities with genuine mRNA translation products. Such DRiPs are widely studied in viral infections and cancer and are considered as the main target of effective immunosurveillance. In human pancreatic islets, we previously showed that cryptic translation initiation sites can be used during translation of insulin mRNA to generate highly immunogenic insulin-gene derived polypeptide targeted by T-cell autoreactivity in T1D patients, providing proof of principle for the participation of translational errors in autoimmune disease(22). Here, our data reinforce our findings and confirm that DRiPs can be generated from human insulin mRNA, even though no significant effect of cytokines could be detected on this particular start site. The specific killing observed in inflammatory milieu despite similar amounts of DRiP polypeptide in cytokine-treated and untreated beta-cells, illustrates the importance of protein stability, degradation and presentation in beta cell destruction(37). Moreover, combining long RNA sequencing and ribosome profiling, we propose another possible explanation to the origin of this immunogenic polypeptide, through the presence of a novel INS-IGF2 transcript that could serve as template for protein synthesis. In line with these findings, the INS-IGF2 locus has been correlated with autoimmunity and loss of beta-cell functionality in T1D(38; 39). Therefore,

deciphering its transcriptional and translational profiling in human islets may be crucial for a better understanding of disease onset and progression.

The large dataset of alternative translato~~me~~ revealed in this study begs for revision of transcriptome or mass spectrometry-based approaches for epitope discovery in autoimmunity, viral infection and cancer. Our data showing ribosome docking at alternative position within the INS transcript and CTL immunoreactivity against the N-term part of the INS DRIP polypeptide, while this epitope could not be eluted from previous peptide elution study (23). Though the human beta cell line are different (ECN90 vs EndoC-BH1/A2) these results emphasize the need to combine transcriptomic/ribosome profiling approaches with immunopeptidomic for epitope discovery. Despite the fact that translation initiation can be regulated by the secondary RNA structure (favoring some transcripts)(40), the results presented here, show that beta-cells may generate up to 22670 novel ORFs potentially immunogenic out of the 15014 start sites identified, assuming a minimal 9-mer residue core for HLA class I peptide. Of note, 9aa ORFs can be presented without further processing as shown for the META~~AA~~VAA peptide derived from a 5'UTR ORF of ARAF protein, eluted as an intact peptide from HLA-B45 in previous study (41) and identified in our database. Although our data largely expand the peptide pool generated by beta-cells and potentially presented at the cell surface, their pathological significance remains to be determined. Certainly, the extreme sensitivity and specificity of CD8 cells to recognize pHLA complexes (42) could explain how the expression of rare peptides can drive an immune response and break tolerance in T1D. This process still remains poorly understood.

Despite remarkable improvement regarding sensitivity, mass spectrometry may not suffice for the identification of short-lived unstable proteins, or for proteins containing many cleavage sites for trypsin that is commonly used in sample preparation. Moreover, biases created by the input

database and low EPCOT scores of out- of-frame sequences may hamper DRIP discovery. Nearly 40% of the overall initiation footprints derived from events of non-canonical translation may generate neo-polypeptides (not in frame sequences), even in resting beta-cells. These findings are consistent with other studies on mouse embryonic stem cells and healthy tissues deriving from hepatocellular carcinoma patients(29; 43). Our data demonstrate that an inflammatory environment led to significant increases in transcripts bearing more than one translation start in beta-cells, alike during tumorigenesis(43). The impact of inflammation on alternative start sites within transcripts encoding for ribosomal subunit proteins (RPL17, RPL35A, RPL37A) may suggest a role in the regulation of the translational process or in the assembly of the ribosome complex that could impact on the generation of immunoribosomes and production of DRiPs(44)(45). In line with other ribosome profiling studies, the 5'-UTR entailed a high proportion of footprints(16; 29; 30; 46). Inflammation induced further upstream translation, highlighting this region as a potentially interesting source for the generation of neoantigens or small regulatory peptides. Besides the difference in amount of uTIS, treatment with inflammatory cytokines caused a shift versus CUG translation compared to the control. Previous studies have shown that CUG translation is mediated by a dedicated Leu-tRNA and EIF2a initiation factor(47), and has been widely correlated with a variety of stressors, like proteotoxic and oxidative stress, viral infection and treatment with Type I and Type II interferons(17; 48). Moreover, CUG initiated polypeptides gave rise to highly immunogenic epitopes (e.g. cryptic antigens originating from VEGFA, bearing an alternative CTG ORFs have already been reported in ENSEMBL database and studied in the context of renal cell carcinoma)(17; 36; 49).

While in our data the classical AUG represent a minority of TIS, these results are in line with previous ribosome profiling studies. Yet, the conservation of a G in -3 and +1 position underlines

the importance of the Kozak consensus sequence in initiating the translation. Recent work aiming at evaluating the strength of start codons has demonstrated that non-AUG codon located within a Kozak-like surrounding sequence may lead to higher translation rates than a non-Kozak AUG(50). Polypeptides arising from these non-AUG alternative starts may not necessarily initiate with non-methionine amino acids since eIF2a methionine carrying tRNA loading on non-AUG starting codon has been provided by in vitro assays using [³⁵S]-labeled Met-tRNA^{Met} (51; 52). Also, some findings from ribosome profiling experiments have been confirmed by mass spectrometry(53), in particular N-term extension of annotated proteins.

Non-canonical TIS were not only present on coding transcripts, we observed that 7% of the footprints originated from various forms of presumably non-coding RNAs, especially lncRNAs. Several studies have confirmed the presence of ribosome attached to lncRNA suggesting the synthesis of small polypeptides (<http://www.sorfs.org/database>). The absence of epitopes in these polypeptides suggests a regulatory function(54).

The study relies on the use of EndoC-βH1 cells as an *in vitro* model for human beta cells. While, those cells have been described to represent the best alternative so far to primary human beta cells and, as such, constitutes a relevant “humanized” model to study beta cell function (in particular EndoC-βH1 cells and human islets have very similar responses to cytokines and other stressors(55-58), recent, in depth multiomics analyses have highlighted some differences between EndoC-βH1 cells and primary beta cells(59)), (e.g. the absence of iNOS expression in response to cytokines(60)), inherent to both the fetal/embryonic origin of the cell line and/or its transformed state. The presence of ribosomes bound to glucagon transcripts in our study may be the reflect of this observation. Another limitation is intrinsic to the ribosome profiling assay, in which a number of identified start sites may be the consequence of monosome footprint or background artifacts.

Yet, despite very stringent detection method (that exclude robust interpretation of ribosome reads at IAPP, GAD65, ZnT8 and IGRP start position), future follow up studies to validate the identified polypeptides, presented here, are required to define the missing fraction of the beta cell proteome. Altogether, our results reveal that the translation process plays a significant role in proteome diversity. While some of these peptides synthesized by beta-cells may prove unstable and lacking biological functions, they may represent by-products of the translation process that can contribute to the beta-cell translome. The fact that translation is highly dynamic and affected by environmental modifications suggests that perturbations occurring on beta-cells at the onset of disease or throughout the course of T1D may change the beta-cell ligandome, generating neoepitopes provoking immune surveillance. Currently, drugs are being developed to inhibit non canonical translation initiation(61), which may offer strategies to control beta-cell homeostasis, immunogenicity and preservation.

Novel non canonical open reading frames warrant further functional and immunological studies. We contend that understanding mechanisms of neoantigen generation is critical for the development of novel immune targeted therapies.

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Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and the Supplementary Materials. Raw datasets are accessible through GEO series accession number GSE167223 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE167223>). All data files (S1-S9) are accessible at [10.6084/m9.figshare.13187714](https://doi.org/10.6084/m9.figshare.13187714).

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Table 1. Primer sequences used to generate the RPF library.

		Primers sequences (5'→ 3')
Size markers	Upper size marker	AUGUACACGGAGUCGAGCUCAACCCGCAACGCGA-(Phos)
	Lower size marker	AUGUACACGGAGUCGACCCAACGCGA-(Phos)
Linker	Universal miRNA cloning linker	rAppCTGTAGGCACCATCAAT–NH2
cDNA	Reverse transcripti on	(Phos)- AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTGC- (SpC18)-CACTCA- (SpC18)- TTCAGACGTGTGCTCTTCCGATCTATTGATGGTGCCTACAG
Purification	rRNA depletion	GGGGGGATGCGTGCATTATCAGATCA
		TTGGTGACTCTAGATAACCTCGGGCCGATCGCACG
		GAGCCGCCTGGATACCGCAGCTAGGAATAATGGAAT
		TCGTGGGGGGCCCAAGTCCTTCTGATCGAGGCC
		GCACTCGCCGAATCCCGGGGCCGAGGGAGCGA
		GGGGCCGGGGCCGCCCTCCACGGCGCG
		GGGGCCGGGCCACCCCTCCACGGCGCG
		CCCAAGTGCCTCCCGGGCGTCGTCGCGCCGTCGGGTCCCGGG
		TCCGCCGAGGGCGCACACCGGCCCGTCTCGCC
		AGGGGCTCTCGCTTCTGGCGCCAAGCGT
		GAGCCTCGGTTGGCCCCGATAGCCGGGTCCCCGT
		GAGCCTCGGTTGGCCTCGGATAGCCGGTCCCCCGC
		TCGCTGCGATCTATTGAAAGTCAGCCCTCGACACA
		TCCTCCCGGGGCTACGCCTGTCTGAGCGTCGCT
Library preparatio n	Forward library PCR	AATGATACGGCGACCACCGAGATCTACAC
	Index reverse library PCR	CAAGCAGAAGACGGCATACGAGATAGTCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCG
		CAAGCAGAAGACGGCATACGAGATACTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCG
		CAAGCAGAAGACGGCATACGAGATATGCTGGTGAAGTTCAGACGTGTGCTCTTCCG
		CAAGCAGAAGACGGCATACGAGATACGTCGGTGAAGTTCAGACGTGTGCTCTTCCG

FIGURE LEGENDS

Figure 1: Ribosome profiling of EndoC-βH1. (A) Schematic representation of experimental workflow. (B) Length distribution of ribosomal footprints of non-treated (control) and IFN γ +IL1 β treated samples (cytokines) (n=3) (C) Venn diagram of P-sites identified in control and cytokines treated samples. The total number of genes carrying a TIS and the total number of TIS are indicated as Σ (D) Qualitative sequence logo of codons composition corresponding to E- (-3 to 0), P-(0 to 3) and A-(3 to 6) pockets of the ribosome.

Figure 2: Mapping of TIS on the transcriptome. (A) Gene ontology plot of canonical TIS (cpm> 0.5), showing significant log fold change compared to the control (logFC>1.5). The plot was generated using clusterProfiler (B) Heatmap of the canonical TIS (cpm>0.5) significantly up- or down-regulated in cytokines treated samples. The heatmap was generated using Pheatmap. (C) Pie charts representing the percentage of alternative and annotated TIS in experimental conditions. (D) Pie charts representing the abundance percentage of the different types of ORFs derived from the alternative TIS represented in C. (E) Pie charts of TIS identified in coding and non-coding RNA. (F) Polar plots of non-coding transcripts subtypes, showing one or more TIS. (G) Length of ORFs potentially generated by the identified TIS. The average length of the ORFs is indicated as μ .

Figure 3: Integration of long RNA sequencing and Ribosome profiling. (A) Mean percentages of ribosomal footprints aligned to the transcriptome (n=3). (B) Nanopore QC analysis showing the RNA read length. The cleaned reads were aligned to the transcriptome (ENSEMBL build GRCh38.99) for the controls (n=3) and cytokine treated samples (n=3) using STAR version 2.7.3a (C) Volcano plot of transcripts expression detected in long RNA sequencing (n=3, read cut off:

mean of replicates=10). T1D susceptible genes are depicted on the figure. **(D)** Venn diagram representing the novel transcripts detected in control and cytokine treated conditions. **(E)** Venn diagram representing the number of TIS detected on novel transcripts (shown in D), upon alignment of Ribosome profiling with long RNA sequencing data. **(F)** Codon usage of TIS on novel transcripts. Sequence logo, corresponding to E- (-3 to 0), P-(0 to 3) and A-(3 to 6) sites of the ribosome pockets. **(G)** Length of ORFs potentially generated by the identified TIS on novel transcripts. The average length of the ORFs is indicated as μ . **(H)** Scatterplot representing the differential expression of the RPF at TIS position (x) versus differential expression of the long RNA sequencing transcripts (y). T1D susceptible genes are depicted on the figure.

Figure 4: Inflammation disturbs the translational process and fidelity. **(A)** Percentage of transcripts showing a unique or multiple TIS (two or more). Statistical analysis was performed using the z-score test. **(B)** Region distribution of the TIS identified on the transcriptome in control or cytokine treated conditions. Statistical analysis was performed using the z-score test. **(C)** Qualitative codon usage profiles of upstream and downstream the canonical start codon. Sequence logo, corresponding to E- (-3 to 0), P-(0 to 3) and A-(3 to 6) sites of the ribosome pockets. **(D)** Volcano plot of the out of frame TIS differential expression per transcript regions. **(E)** Scatterplot representing the differential expression of the RPF at canonical TIS position versus differential expression of the out of frame alternative TIS. Spearman correlation test was used to calculate the R factor and the corresponding P-value. T1D susceptible genes are labelled on the figures.

Figure 5: TIS and aTIS within INS transcripts. **(A)** TIS positioned on the INS transcript (ENST00000381330). The red arrow indicate the position of the INS₂₈₆. Primers used for RT-PCR

are indicated as primer set 1. **(B)** Schematic representation of the INS, INS-IGF2 transcripts detected by long RNA sequencing. Newly identified transcripts are labelled as “TALONT”. The arrows indicate the canonical start site (black) and the INS₂₈₆ (red) (left panel). RT PCR performed on EndoC-βH1 RNA and primary islet isolated RNA using TALON specific primers and sequence analysis of the PCR amplicon **(C)** TIS mapping of the INS-IGF2₉₅ TIS on the novel INS-IGF2 transcript, TALONT000499153. The INS-DRIP epitope is underlined. The sequence in red show the new potential HLA-A2 epitope predicted from the INS-IGF2 polypeptide. **(D)** Killing assay performed on JY cells loaded with PPi peptide, B chain peptide and DRiP peptide using DRiP specific CD8 T cells. T cell activation is shown as CD107a staining (left panel) and MIP1β release (right panel). **(E)** HLA-A/B/C expression in EndoC-BH1/HLA-A2 upon treatment (upper panel) and HLA-A2 expression in EndoC-BH1 and EndoC-BH1/HLA-A2 cells (lower panel). **(F)** EndoC-βH1 and EndoC-βH1 /HLA-A2 coculture with DRiP specific CD8 T cells after incubation with cytokines (IFNγ and IL1β) for 24h (n=3 independent experiments). T cell activation is shown as CD107a staining (left panel) and MIP1β release (right panel).

SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Harringtonine treatment optimization. (A) Example of the bell shaped curve read distribution encountered at TIS locations (GAPDH transcript). (B) Metaplots of reads distribution around the annotated start and stop codons after 2' (upper) or 30' (lower) harringtonine treatment (C) Metaheatmaps of the density of extremities of sequenced footprint fragments around the annotated start after 2'(upper) or 30'(lower) harringtonine (D),(E) IGV browser screenshots of reads mapping on GAPDH and ACTB genes after 2'(upper) or 30'(lower) harringtonine. The small boxes depict the UTRs, the large boxes the CDS, and the dashed lines the intronic region.

Figure S2: Enrichment algorithm. (A) Schematic representation of enrichment algorithm applied on the Ribowaltz output. (B) Example for HLA-B transcripts showing potential TIS distribution before (upper) and after (lower) applying the enrichment algorithm. The orange bar colour indicate an increased enrichment upon cytokine stimulation.

Figure S3: Protein validation. CHGA, STAT1, STAT2, PSMB8, PSMB9 and PSMB10 expression level in EndoC- β H1 cells (A) and primary human islets (B) after exposure to cytokine (IFN γ and IL1 β). Protein quantification is performed using ImageJ and shown as relative expression using actin as reference. n.d. indicate no detected band. *indicate the immunoproteasome subunit position. The blots shown are representative of 2 independent experiments (2 islet preparations).

Figure S4: Examples of TIS mapping. (A),(B) TAP1 and PSMD8 TIS profiling. x axes represent transcript location. X=0 shows the annotated translation start. Arrows indicate the detected main TIS, consistent with the TIDdb database. Orange bars show TIS significantly upregulated upon cytokine treatment. (C),(D) YBX1 and PGRMC1 TIS profiling. x axes represent transcript location. X=0 shows the annotated translation start. Arrows indicate TIS detected by mass spectrometry(53). The orange bars show TIS significantly upregulated upon cytokine treatment. Internal in frame TIS are not depicted in all graphs. (E,F) TIS mapping on CHGA and VEGF transcripts. The orange bars show TIS significantly upregulated upon cytokine treatment. The ORFs' amino-acid sequence based on the upregulated TIS are shown in the table below. The aa highlighted in red indicate potential HLA-A2 binder based on NetMHCI prediction. x axes represent transcript location. X=0 shows the annotated translation start.

Figure S5: Validation of the *de novo* transcriptome. A) Venn diagram showing the number of transcripts (table S4) supported by reads in splicing junction. B) Similar representation for T1D susceptible transcripts. From 6892 TALON transcripts, 3425 novel intronic junctions (and 212 novel intronic junctions in T1D susceptible genes specifically) were detected out of the 2305 intronic containing transcripts. By comparing datasets, 2550 intronic junctions (163 out of 212 novel introns detected in all T1D transcripts) were detected by Illumina bulk RNAseq. C) RT PCR performed on EndoC-βH1 RNA and primary islet isolated RNA using TALONT ALONT000709680 specific primers for detection of PSMB9 transcript variant. D) RT PCR performed on EndoC-βH1 RNA and primary islet isolated RNA using TALONT000700063 specific primers for detection of HLA-C transcript variants.

Figure S6: Inflammation disturbs the translational process and fidelity. Volcano plot representing the differential read expression at start site position in newly identified transcripts (**A**) and within non-coding RNA (**B**). T1D susceptible genes are depicted on the figures.

Figure S7: Pathway analysis of alternative start sites more upregulated than the corresponding canonical starts. Analysis performed by Stringdb at medium confidence and two k-mean clusters. The red nodes show proteins of the mRNA processing pathway, and the red-blue nodes show proteins specifically involved in mRNA splicing and located in stress granules. The combs colouring is based on the default String settings.

Figure S8: Characterization of the CHGA 5'-UTR region. A) Representation of the (Alt-CHGA)-GFP fusion construct. The 5'-UTR region of CHGA is represented as white box; the GFP ORF deleted from the AUG is shown as dark blue box; The CHGA canonical start site and its ORF are presented in blue. The Alt-CHGA-GFP ORF is represented in green and position of the in frame start sites -103; -40 and -22 are shown with black arrow. B) sequence of the (Alt-CHGA)-GFP construct. C) GFP FACS profile of HEK 293 cells transfected with the (Alt-CHGA)-GFP or GFP (with AUG) constructs. D) Western blot analysis showing expression of the GFP fusion proteins after transfection in HEK 293 cells and cytokine stimulation. 0.5ug protein loaded of CMV-GFP transfected cells; 50ug protein loaded on of (Alt-CHGA)-GFP transfected cells. NT: non transfected; MW: molecular weight showing the bands at 37kDa and 25kDa. Upper panel stained with anti-GFP antibody (* show unspecific band); Lower panel stained with anti-Actin antibody (uncut blots are presented as supplementary material)

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure S1.

A.

B.

C.

D.

E.

Figure S2.

Supplementary Figure S3

Figure s4.

Supplementary Figure S5

Figure S6.

Figure S7.

Figure S8.