Identification of islet-enriched long non-coding RNAs contributing to β-cell failure in type 2 diabetes

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1. INTRODUCTION

Type 2 diabetes (T2D) is characterized by reduced insulin action and/or insulin deficiency [1,2]. Insulin is responsible for the control of blood glucose levels and its release is finely tuned by nutrients, hormones, and neurotransmitters. Under obese conditions, a major risk factor for T2D, the peripheral tissues become less sensitive to insulin [3]. To maintain euglycemia and overcome peripheral insulin resistance, β-cells expand and increase their secretory activity [3]. However, in genetically predisposed individuals, defects in this process can lead to progressive deterioration of β-cell function and loss of β-cell by apoptosis, promoting the development of diabetes [3−5]. The mechanisms underlying compensatory β-cell mass expansion, β-cell failure and progression of diabetes are still largely unknown. The proposed causes of β-cell failure include, mitochondrial dysfunction, oxidative and endoplasmic reticulum stress, dysfunctional triglyceride/free fatty acid cycling, and glucolipotoxicity [3,5]. These phenomena trigger alterations in the level of key protein-coding genes and microRNAs [3,6,7], a class of small non-coding RNAs playing central roles in numerous physiological and pathological processes [8]. Beside protein-coding genes and microRNAs, transcriptome analysis identified another large class of non-coding RNAs, the long non-coding RNAs (lncRNAs) [9−12]. These non-coding transcripts participate in diverse gene-regulatory mechanisms [13−15] and their dysregulation has been implicated in many human diseases [16]. Recently, lncRNAs were found to contribute to β-cell development and glucose homeostasis [17,18] and to be involved in β-cell demise during the initial phases of type 1 diabetes [19,20]. However, so far only very few lncRNAs have...
been functionally characterized and very little is known about their possible contribution to T2D development. The aim of this study was to identify novel islet IncRNAs and to investigate their role in the regulation of β-cell functions. The expression of IncRNAs is highly cell-type and context specific. In view of this, we used RNA-sequencing to explore unbiasedly the islet transcriptome for novel IncRNAs expressed in mice fed a high-fat diet, a model of mild diabetes associated with obesity that corresponds to early diabetes in human [21]. We identified several not yet annotated IncRNAs, a number of which displayed expression changes between islets of lean and obese mice. Analogous changes of selected IncRNAs were also observed in islets of obese diabetic db/db mice and in the islets of T2D donors. In addition, the modulation of some of these IncRNAs in dissociated mouse islet cells sensitised the β-cells to apoptosis. Overall, the results show that IncRNAs are modulated in islets from obese diabetic mice and T2D individuals and may contribute to β-cell failure during T2D development.

2. MATERIAL AND METHODS

2.1. Chemicals
IL-1β, leptinotycin B, collagenase, and Histopaque were purchased from Sigma—Aldrich (St Louis, MO, USA), TNF-α from Enzo Life sciences (Farmingdale, NY, USA) and IFN-γ from R&D systems (Minneapolis, MN, USA).

2.2. Animals
Five-week old male C57BL/6 mice (Charles River Laboratories, Raleigh, NC, USA) were fed a normal (ND) or a high-fat diet (HFD) for 8 weeks (Biover F-3282, 60% energy from fat, Farmington, NJ, USA) [21]. The animals on high fed diet were subdivided in low (LDR) and high responders (HDR) according to the criteria defined in Peyot et al., 2010 [21]. The mice in the LDR group weighted between 33 and 39 g after 7.5 weeks on HFD while the animals in the HDR group between 39 and 45 g. C57BL/KsJ db/db mice (13–16 weeks) and age-matched lean db/+ littermates were obtained from the Garvan Institute breeding colonies (Sydney, NSW, Australia) [22]. Mice expressing the enhanced yellow fluorescence protein in β-cells (RIPYY) were obtained by crossing ROSA26-YFP with RIP-Cre mice [23]. Animal procedures were performed in accordance with National Institutes of Health guidelines and were approved by research councils and veterinary offices.

2.3. Islet and insulin-secreting cells
Mice islets were isolated by collagenase digestion followed by Histopaque density gradient [24] and cultured in RPMI medium [19]. Islets from RIPYY mice were either directly used to isolate the RNA or dissociated to separate β- from non-β cells by FAC-sorting [23]. Human islets were provided by the Cell Isolation and Transplantation Center (University of Geneva) or by the Human Tissue Lab of EXODIAB/Lund University Diabetes Centre through the Nordic Network for Islet Transplantation, Uppsala University. After isolation, the human islets were utilized for RNA isolation or cultured in CMRL medium (Invitrogen) supplemented with 10% FCS, 100 μg/mL streptomycin and 100 IU/mL penicillin (Invitrogen), 2 mmol/L L-glutamine and 250 μmol/L HEPES. Dissociation of mouse and human islets was achieved by incubation at 37 °C in PBS containing 3 mM EGTA and 0.002% trypsin. MIN6B1 cells were cultured in DMEM-Glutamax medium (Invitrogen) [25].

2.4. RNA-sequencing and analysis
RNA was isolated using the RNeasy kit (Qiagen), followed by DNase treatment (Promega). Ribosomal RNA was removed using the Ribog Zero Magnetic Gold kit (MRZG126, Illumina), and sequence libraries were prepared using the Illumina TruSeq stranded mRNA LT kit without poly(A) selection in order to include also all the IncRNA transcripts that are not polyadenylated. Libraries were sequenced with the Illumina HiSeq2000. 100nt paired-end reads from 6 samples were mapped to mm9 reference genome using Tophat software version 2.0.8 [26] with the option "Gene model"—G, using mm9 UCSC reference genes GTF [27]. Ab initio transcript reconstruction was performed using Cufflinks, version 2.1.1 [26], with option —G and the reference UCSC genome. The resulting GTFs were merged using Cuffmerge v2.1.1 [28] to distinguish known and novel transcripts. Using the output of Cuffmerge, the transcripts were divided into 3 categories: known mRNAs, known IncRNAs (UCSC as reference), and novel IncRNAs. Novel transcripts were filtered for having at least 2 exons. Read counts were then calculated per gene from the alignment bam files using HTSeq (v0.5.4p3) with options —m union—stranded no. Genes were then filtered for minimal expression (mean counts \( \geq 5 \) across all conditions). The protein-coding potential of transcripts was evaluated using the program GeneID [29], v1.4.4, applied to transcript sequences in FASTA format, with parameters adapted for vertebrates as provided by the authors in file GeneID.human.070123.param and with options —s and —G. Transcripts with a coding potential \( >4 \) were removed from the analysis. Differentially expressed genes were detected using the limma package in R by first transforming the raw count data to log2 counts per million reads using the voom function. Empirical Bayes moderated \( t \) statistics and corresponding \( p \)-values were computed for the comparison and \( p \)-values adjusted for multiple comparisons using the Benjamini-Hochberg procedure [30]. Genes with an adjusted \( p \)-value of \( \leq 0.05 \) were considered differentially expressed. Differential analysis by transcripts was done using Cuffdiff, v2.1.1 [28], on a gtf file containing the coordinates of the novel transcripts. Gene ontology analysis was performed by submitting the genes lists to the DAVID Functional annotation clustering tool using default parameters (https://david.ncifcrf.gov/tools.jsp).

2.5. Measurement of IncRNAs expression
RNA was reverse transcribed using M-MLV reverse transcriptase, RNAse H minus (Promega). Quantitative PCR was performed using qSYBR Green mix and samples were amplified using the CFX Connect Real-time system (Bio-Rad). Islets of human control and T2D patients were homogenized by vortexing in 700 μL Qiaozal lysis buffer and the RNA extracted using the miRNeasy kit (Qiagen) with DNase treatment. 100ng total RNA was used for reverse transcription using the High Capacity cDNA kit with RNAse inhibitor (ThermoFisher). For qPCR, PowerUP SYBR Green Master Mix (Applied Biosystems) was used with assay-specific primers (Supplemental Table 1).

2.6. Subcellular fraction
MIN6B1 cells were incubated for 15 min in ice-cold lysis buffer (10 mM Tris–HCl, pH 7.5, 0.05% NP40, 3 mM MgCl2, 10 mM NaCl and 5 mM EGTA) and then centrifuged 10 min at 2,000 \( \times g \). The supernatant (cytoplasmic fraction) was recovered while the pellet was resuspended in 10 mM HEPES, pH 6.8, 300 mM sucrose, 3 mM MgCl2, 25 mM NaCl, 1 mM EGTA, 0.5% Triton-X-100 and treated with 700 U/ml DNase I for 30 min at 4 °C. The samples were then centrifuged at 17,000 \( \times g \) for 20 min and the pellet collected as nucleoplasmonic fraction.

2.7. Transfection
Overexpression of IncRNAs was achieved by transfecting pcDNA3-based plasmids with Lipofectamine 2000 (MIN6B1 cells) or 3000 (primary cells) (Invitrogen). Down-regulation was carried out by
transfecting Gapmers (Exiqon) with RNAiMax (Invitrogen) (gapmers sequences in Supplemental Table 1).

2.8. Insulin secretion
Insulin secretion of MIN6B1 cells was carried out as described [25].

2.9. Assessment of cell death
Apoptotic cells displaying pyknotic nuclei were scored under blind conditions by fluorescence microscopy (AxioCam MRc5, Zeiss, Feldbach, Switzerland) after incubation with 1 μg/ml Hoechst [25] or TUNEL assay (Roche).

2.10. NF-κB nuclear translocation
MIN6B1 cells were transfected with a plasmid expressing a GFP-tagged form of NF-κB subunit p65 (Rela) and/or the plasmid expressing the lncRNA. 24 h later, the cells were treated with the indicated compounds for 3 h, fixed, and mounted on a coverslip for microscopic examination.

2.11. Statistical analysis
Data are presented as mean ± sem. Statistical differences were assessed by two-tailed paired Student’s t-test when only two sets of data were present or by one-way ANOVA with more than 2 groups with a discriminating p-value of 0.05 (GraphPad Prism). Correlations between lncRNA expression and different characteristics of the individuals were performed by linear regression, where F-test was used to determine significance at p < 0.05.

ACCESION NUMBERS: RNA-sequencing data have been deposited in the Gene Expression Omnibus Database, accession number GSE92602.

3. RESULTS
To investigate the contribution of IncRNAs to β-cell dysfunction and T2D development, we compared the transcriptome of islets from mice fed a regular and a high-fat diet. The metabolic characteristics of these animals are summarized in Supplemental Table 2. RNA-sequencing yielded ~500,000,000 reads per sample (accession number GSE92602), of which, ~75% were mapped to the mouse genome. *Ab initio* transcript assembly was performed using Cufflinks [26], and novel transcripts were classified from known IncRNAs and protein-coding mRNAs. Hierarchical clustering showed a distinguishable expression profile between the two groups (Figure 1A). The analysis detected 14874 protein-coding genes, of which, 971 were upregulated and 395 downregulated in mice fed a high-fat diet (Figure 1B and Supplemental Table 3). Functional annotation of the differentially expressed protein-coding genes showed enrichment for biological pathways related to protein localization and transport, redox processes, intracellular transport (Supplemental Fig. 1). Of these differentially expressed genes, 21.2% overlapped with those previously identified by microarray [31]. We also detected 1761 UCSC annotated IncRNAs (23 upregulated and 104 downregulated) and 1996 non-annotated UCSC IncRNA genes (4303 transcripts), of which, 39 were upregulated and 107 downregulated (Figure 1B, Supplemental Tables 4 and 5). Amongst the non-annotated IncRNAs, 438 overlapped with recently published transcripts [32,33] while 1558 were novel. We compared our mouse data with those of Moran et al. [34] obtained in human using Transcript, a cross species mRNA alignment tool. A list of mouse IncRNA genes for which we were able to identify the corresponding human orthologues is provided in the Supplemental Table 6. The GenelD-coding potential score revealed that our novel lncRNA candidates have minimal protein-coding potential (Figure 1C). The expression level of the novel IncRNAs overlapped that of UCSC-annotated IncRNAs and was usually lower than that of protein-coding genes (Figure 1D). The coordinates of all newly annotated transcripts is available on GEO (accession number GSE92602).

We then used different criteria to select candidate IncRNAs for further analysis, including clearly detectable expression changes in response to high-fat diet, the presence of a small number of isoforms to avoid dealing with several overlapping transcripts with potentially different functions, and the putative presence of human orthologues. Multidimensional analysis revealed that the transcriptome of one of the animals (HDR3) was slightly different from the other mice on high-fat diet (Supplemental Fig.2). To avoid missing potentially interesting candidates, we included in our initial screening also the IncRNAs showing significant differences between control mice and the other two mice on HFD (Supplemental Table 7). We selected two intergenic IncRNAs (XLOC_010971 and XLOC_013310) for further analysis. The chromosomal location and the fold changes of these two novel IncRNAs are shown in Figure 1B. Since there is not yet a consensus for the nomenclature of mouse IncRNAs, they are hereafter referred to as βlinc2 and βlinc3 (β long intergenic noncoding RNA 2 and 3) to follow Arnes et al. nomenclature [18]. The locus architecture, the number of isoforms, and the coding potential of βlinc2 and βlinc3 are provided in Figure 1E–F and in the Supplemental Table 8. Subcellular fractionation of MIN6B1 cells revealed that βlinc2 is present both in the cytosolic and in the nuclear fractions while βlinc3 is essentially nuclear (Supplemental Fig. 3).

The changes observed by RNA-sequencing were confirmed by qRT-PCR in additional samples of high responders to the high-fat diet (HDR), as well as in low responders (LDR), a group of animals displaying the phenotypic characteristic of pre-diabetes when compared to obese humans [21]. The level of βlinc2 was not increased in the LDR group with no glycemical alterations but was upregulated 49 times in the HDR group that shows mild hyperglycemia [31]. The expression of βlinc3 tended to decrease already in the LDR group but reached significance only in the HDR group that displayed 60% lower levels compared to controls (Figure 2A–B). The expression of these transcripts was also analyzed in the islets of db/db mice, which lack the leptin receptor and develop severe obesity associated with T2D [22,35]. The increase of βlinc2 was more pronounced than in HDR mice, with an up-regulation of 1802-fold, whereas the decrease in βlinc3 expression was similar to that observed in response to high-fat diet HDR group (Figure 2C–D).

We then tested whether the expression of these two IncRNAs correlated with body weight, glycemia, and insulinemia of animals fed normal and high-fat diets (metabolic characteristics are provided in Supplemental Table 9). As shown in Figure 3 A, C, E we found a positive correlation between the level of βlinc2 with body weight, glycemia, and insulinemia. This was true even when performing the analysis after the exclusion of the highest point (Supplemental Fig. 4). The raise of βlinc2 was mainly observed in animals weighing >40 g, suggesting the existence of a threshold. In contrast, the expression of βlinc3 was negatively correlated with these parameters except for insulinemia (Figure 3B,D,F).

To assess whether the expression of these two novel IncRNAs is restricted to pancreatic islets, we analyzed their levels in a large panel of tissues. We found that βlinc2 is undetectable in the investigated tissues except in heart, where the expression is about 100 times lower compared to islets (Figure 4A). βlinc3 was only detectable in heart and brain, but again at much lower levels compared to islets (Figure 4B). To
Figure 1: Overview of the RNA-sequencing results. A. Hierarchical clustering of samples using the 500 genes displaying the highest mean expression. Colors display Euclidian distance, red represents no distance, yellow means there is a longer distance. ND, normal diet; HDR, high-(high fat diet) responders. B. Summary of differentially expressed genes (up right) and studied lncRNAs with fold changes and p-values (bottom right). C. Coding potential for novel transcripts compared to known coding genes. The red line represents the cutoff used to filter and classify the novel transcripts (<4, GeneID). D. Size distribution of protein-coding genes, known and novel lncRNAs. E. Locus architecture and isoforms of the bllinc2 gene. F. Locus architecture and isoforms of the bllinc3 gene.
Figure 2: The expression levels of two lncRNAs are modulated in islets from mice fed a high fat diet and in db/db mice. Expression of linc2 and linc3 in C57BL/6 mice fed a normal diet (ND) and in low–high fat diet responders (LDR) and in high–high fat diet responders (HDR) (A,B) and from db/+ and db/db mice (C,D). Islets were isolated from mice of 14 weeks of age after being fed a standard or a high fat diet for 8 weeks and from db/db mice of 13–16 weeks of age. The expression levels of the lncRNAs were measured by real-time PCR and normalized to Gapdh. t-test or ANOVA, Kruskal–Wallis post hoc test, *P < 0.05, **P < 0.01, ***P < 0.001 vs ND or db/+.

assess whether these lncRNAs are expressed in insulin-secreting cells, we measured their level in highly purified (~99%) β-cell fractions obtained from FAC-sorted islet cells [23]. We found that linc2 and linc3 are indeed abundant in the β-cell fraction (Figure 4C–D). To determine the possible causes of the changes in lncRNA expression detected in the islets of obese mice, we exposed normal mouse islets to pathophysiological concentrations of glucose and palmitate. The expression of linc2 increased in the presence of high glucose (20 mM) and palmitate (0.5 mM), whereas linc3 was only modified by the presence of palmitate (Figure 5). These changes were not observed upon incubation of dissociated islet cells with pro-inflammatory cytokines (Supplemental Fig. 5).

Subsequently, we searched for human orthologues of these two lncRNAs. We mapped the identified mouse lncRNA sequences to the human genome using TransMap. To validate the predicted orthologues we designed primers in the putative exons. This enabled us to detect human lncRNAs. We mapped the identifications of specific β-cell functions, we modified their level in MIN6 and mouse islet cells. Overexpression of linc2 and down-regulation of linc3 in MIN6 cells (Supplemental Fig. 6) did not modify proinsulin mRNA levels, insulin content, or insulin release (Supplemental Fig. 7). As an increase in β-cell apoptosis and a consequent reduction in the β-cell mass can contribute to T2D development [4], we investigated the impact of changes in the expression of these lncRNAs on β-cell survival. We observed that the up-regulation of linc2 increases apoptosis of both MIN6 (Figure 8A) and dissociated mice islet cells (Figure 8C) to a level similar to the one seen upon 24 h exposure to pro-inflammatory cytokines (used as a positive control for apoptosis). Overexpression of linc3 did not affect cell survival (Figure 8A–C). However, downregulation of linc3 in MIN6 and dissociated mice islet cells, mimicking the conditions encountered under diabetic state, resulted in a rise in the number of apoptotic cells (Figure 8C–D). Similar results were obtained by TUNEL assay and using a different gapmer targeting linc3 (Supplemental Fig. 8 and 9).

Increasing evidence suggests an involvement of inflammatory processes in the pathogenesis of T2D and activation of the NF-κB pathway has been implicated in glucolipotoxic-induced β-cell apoptosis [36–38]. Among other mediators, hyperglycemia and hyperlipidemia increase the production and the release of IL-1β, a pro-inflammatory cytokine that activates the transcription factor NF-κB [42]. As one of the initial events occurring shortly after β-cell exposure to IL-1β is the translocation of NF-κB to the nucleus [40], we transfected MIN6 cells with a GFP-tagged form of p65 (the main NF-κB subunit) [39,41] and monitored its subcellular distribution after modulating the level of the lncRNAs. We found that incubation of the cells with leptomycin B, a compound that impedes the nuclear exit of NF-κB [42] was sufficient to increase the fraction of cells in which p65 was localized in the nucleus (Figure 8E). As expected, the localization of p65 in the nucleus was further increased when incubating the cells with high concentrations of IL-1β. No difference was seen by...
overexpressing \( \beta \text{linc}2 \) alone, but, in the presence of leptomycin B, we observed an increase in the number of cells in which \( \text{p65} \) is localized in the nucleus similar to the one seen in the presence of IL-1\( \beta \) (Figure 8 and Supplemental Fig. 10). These data suggest that at least part of the effect of \( \beta \text{linc}2 \) on \( \beta \)-cell survival may be related to an increased shuttling of NF-\( \kappa \)B to the nucleus. No differences were seen when up-regulating (Supplemental Fig. 11) or down-regulating \( \beta \text{linc}3 \) (data not shown). We then assessed whether the changes in the level of \( \beta \text{linc}2 \) or \( \beta \text{linc}3 \) are directly affecting the expression of key apoptotic genes. As shown in Supplemental Fig. 12, none of the tested mRNAs was modified upon overexpression of \( \beta \text{linc}2 \) or down-regulation of \( \beta \text{linc}3 \).

4. DISCUSSION

Human islets have recently been shown to express a large number of lncRNAs that, in concert with transcription factors, regulate the transcriptional landscape of \( \beta \)-cells [34,43]. In this study, we used high-throughput RNA-sequencing to identify novel lncRNAs modified in a mouse model of diet-induced obesity and hyperglycemia that are potentially involved in the control of \( \beta \)-cell functions and \( \beta \)-cell failure. This T2D model integrates both genetic and environmental risk factors. Amongst the mice fed a HFD, the high responders (HDR) were chosen for the initial analysis since they correspond to the early diabetes situations in humans and display phenotypic features such as insulin resistance, hyperinsulinemia and hyperglycemia [21], typically encountered during the development of T2D.

The capacity to identify new transcripts is strongly influenced by the length and the depth of the sequencing and is more efficient if the sequencing is paired-ended. Our transcriptomic analysis was carried out with an unprecedented depth (100 nucleotide paired-end sequencing and 500 million reads per sample) and included also RNAs lacking a polyA tail. For comparison, in the other two main studies devoted to the identification of novel lncRNAs in mouse \( \beta \)-cells, Benner et al. [32] generated 30 million single reads per sample and Ku et al. [33] 150—371 million 82—85 paired-end sequencing reads per sample, resulting in the identification of 127 and 1359 non-annotated lncRNAs, respectively. Our comprehensive analysis led to the discovery of many novel transcripts with minimal protein-coding potential. This is in line with the view that lncRNA expression is more cell- and context-specific compared to that of protein-coding genes [11]. In agreement
with other reports [11,34], the level of the newly annotated islet IncRNAs was lower than that of protein-coding genes and overlapped that of previously annotated lncRNAs. We found that the expression of many of the newly annotated IncRNAs is modulated by the diet. We focused on two lncRNAs that are highly enriched in β-cells compared to other tissues. We found that the expression of these IncRNAs is altered also in 13–16 week-old diabetic db/db mice. The down-regulation of βlinc3 was similar in both models, whereas the expression of βlinc2 was more drastically affected in the islets of db/db mice than in HDR samples. This difference is associated with a more severe phenotype of β-cell failure and diabetes displayed in db/db mice compared to high-fat-fed mice [35].

The expression of βlinc2 was also positively correlated to body weight, glycemia, and insulinemia in ND and a HFD mice. However, its level was not significantly increased in the islets of low diet responder mice (LDR), suggesting that the changes may only occur under severe obesity and insulin resistant conditions with associated hyperglycemia. βlinc3 expression was negatively correlated to body weight and glycemia but not to insulinemia, suggesting that the decrease of this lncRNA is mainly associated with the development of obesity and less with the control of insulin release.

During the development of obesity-associated T2D, β-cells are chronically exposed to glucolipotoxic conditions [44]. Hence, we treated mouse islets with increasing concentrations of glucose or with the free fatty acid palmitate to investigate whether these pathophysiological conditions may explain the changes in IncRNA expression. Indeed, the level of βlinc2 was increased by both elevated glucose and palmitate concentrations, whereas βlinc3 was down-regulated by palmitate but not by glucose.

LncRNAs are less conserved than protein-coding genes [11] and may have implications in species-specific functions [45]. To confirm the relevance of our findings for human diabetes we search for potential orthologues. Unfortunately, although alignment tools indicated the existence of potential candidate regions, we could not formally identify a human orthologue of βlinc2. The function of lncRNAs can be preserved with sequence homologies as low as 21% [46]. Thus, computational tools based solely on sequence alignments may be inappropriate for the identification of lncRNA orthologues. In the future, a better understanding of the mode of action of lncRNAs will hopefully promote the design of new tools to search for orthologues facilitating the identification of human transcripts with functions analogous to mouse βlinc2.

Using the available tools, we were able to identify the orthologue of βlinc3 in human islets and to confirm that its levels are also diminished upon treatment in the presence of elevated concentrations of palmitate. Since exposure of islet cells to elevated palmitate in vitro induces more rapid and harmful effects than those that may occur in vivo, we measured the level of the human βLINC3 in islets of control and T2D donors. Interestingly, the level of βLINC3 was lower in subjects with T2D, and, as was the case in mice, there was a negative correlation between its expression and the BMI of the subjects.
Figure 5: In vitro effects of chronically-elevated glucose and palmitate on the level of two lncRNAs differentially expressed in islets from mice fed a high fat diet. Isolated islets from C57BL/6 mice fed a regular chow diet were incubated for 48 h at 6, 11 or 20 mM glucose and at 6 mM glucose with or without 0.5 mM palmitate (RPMI supplemented with 5% FCS, 0.5% BSA and 11 mM glucose). LncRNA expression was measured by real-time PCR and normalized to Gapdh. Means ± SEM of 3–4 different experiments. t-test or ANOVA, Tukey post-hoc test. *P < 0.05 vs control, either glucose 6 mM or no palmitate.

Figure 6: Effects of chronically-elevated glucose and palmitate on the levels of βLINC3 in human islets. Human islets were incubated for 48 h at 5.5, 10 or 20 mM glucose, and at 5.5 mM glucose with and without 0.5 mM palmitate (CMRL, 5% FCS, 0.5% BSA, 5.5 mM glucose). LncRNA expression was measured by real-time PCR and normalized to GAPDH. Means ± SEM of 3 different experiments. *P < 0.05 vs control, either glucose 5.5 mM or no palmitate.
Moreover, there was a trend for a negative correlation between the levels of \( \beta \text{LINC3} \) and HbA1c, suggesting that lower amounts of \( \beta \text{LINC3} \) may result in poorer glycemic control. The analysis of more subjects would be needed to confirm this assumption.

In genetically predisposed individuals, the progression of the disease coincides with a gradual deterioration in \( \beta \)-cell functions, in part associated with the loss of \( \beta \)-cells by apoptosis [3,5]. The modulation of these two lncRNAs had no impact on insulin biosynthesis or release, but the increase of \( \beta \text{linc2} \) and the decrease of \( \beta \text{linc3} \) resulted in a rise in the number of apoptotic cells. Thus, altered expression of these lncRNAs cannot explain the secretory defects observed in \( db/db \) or HFD mice but may contribute to \( \beta \)-cell failure during the development and the progression of the disease.

The precise mechanisms underlying the effect of \( \beta \text{linc2} \) and \( \beta \text{linc3} \) on \( \beta \)-cell apoptosis remains to be determined. We found that overexpression of \( \beta \text{linc2} \), increases the rate of NF-\( \kappa \)B nuclear translocation. Although basal NF-\( \kappa \)B activity is required for normal insulin release [47], its prolonged activation plays a central role in cytokine-mediated inflammatory events including the speed of the nuclear translocation of the transcription factor and/or a more sustained activation of this pathway [37,40]. One of the initial events leading to the activation of NF-\( \kappa \)B, for instance in response to IL-1\( \beta \) [40], a cytokine produced in conditions of hyperglycemia and hyperlipidemia [48,49], is its translocation to the nucleus. Since the extent of the induction of NF-\( \kappa \)B target genes is influenced by different events including the speed of the nuclear translocation of the transcription factor and/or a more sustained activation of this pathway [39,40], it is possible that an increased shuttling of NF-\( \kappa \)B to the nucleus may promote the activation of this signaling cascade ultimately contributing to apoptosis. Despite the increased shuttling of p65 to the nucleus alone may not be sufficient to induce the expression of the target genes and may require additional convergent signals. Since \( \beta \text{linc2} \) is not induced by pro-inflammatory cytokines, the effect of the lncRNA on NF-\( \kappa \)B cannot reflect the release of IL-1\( \beta \) from \( \beta \)-cells. Thus, the induction of \( \beta \text{linc2} \) is likely to occur through a different, yet to be identified, mechanism. This alternative pathway may potentially synergize with the canonical cytokine-induced pathway leading to a more drastic activation of NF-\( \kappa \)B. Additional studies will be needed to elucidate the mode of action of \( \beta \text{linc2} \) and \( \beta \text{linc3} \) and to dissect the molecular events through which these lncRNAs can affect the survival of \( \beta \)-cells. The lncRNAs Lethe and Nkila have been shown to interact directly with components of the NF-\( \kappa \)B pathway [50,51]. Future studies should determine whether this is also the case for \( \beta \text{linc2} \).

5. CONCLUSION

The discovery that mammalian genomes are extensively transcribed and generate thousands of transcripts lacking protein-coding potential has opened new perspectives in the study of the mechanisms regulating the activity of \( \beta \)-cells and of the causes of their failure under diabetic conditions. We have identified a large number of novel lncRNAs many of which are modulated under obesity and T2D conditions. At least two of them can affect the survival of \( \beta \)-cells and may potentially contribute to glucolipotoxic-mediated \( \beta \)-cell loss and to the manifestation and progression of T2D. The genome-wide data obtained in this study will provide the basis for future investigations on the involvement of other novel and potentially islet-specific lncRNAs in \( \beta \)-cell dysfunction and T2D development. This may pave the way to the identification of new therapeutic targets for diabetes prevention and treatment. Indeed, lncRNAs display a more restricted tissue distribution than protein-coding genes [11,34], providing the ideal targets for highly specific therapeutic interventions. Examples already exist where...
the expression of these transcripts was manipulated in vivo in mice [17,52].

**AUTHOR CONTRIBUTIONS**

AM conceived the experiments, generated the research, analyzed the data, wrote the manuscript, and approved its final version. SG, MLP, DRL, JLSE, AGR, FB, MI, LE, PG, and MP contributed to the acquisition of data, reviewed the manuscript, and approved its final version. RR conceived the experiments, analyzed the research data, wrote the manuscript, and approved its final version.

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Figure 8: Overexpression and downregulation of the lncRNAs b.linc2 and b.linc3 promotes apoptosis in MIN6 β cells. (A) MIN6 cells were transfected for 48 h with a control plasmid (vector) or plasmids to induce the overexpression of the lncRNAs of interest. (B) The cells were transfected with a control gapmer or a gapmer targeting b.linc3 to knockdown this particular lncRNA. After 24 h of incubation, some of the cells were exposed to a mix of cytokines [0.1ng/ml IL-1β, 10ng/ml TNF-α and 30 ng/ml IFN-γ] as a positive control. The same experiments were repeated in dispersed mouse islet cells (C, D). The proportion of cells showing pyknotic nuclei was scored in at least 600 cells for condition. Fold changes were calculated by dividing the results by the values obtained in the control condition. The data represent the means ± SEM of 3 – 6 different experiments. E. MIN6 cells were transfected for 48 h with a plasmid expressing GFP-tagged p65 (Rela). They were then exposed for 3 h to either a high dose of IL-1β (10ng/ml), Leptomycin B (LMB) (22ng/ml) or a combination of the two. The number of cells displaying nuclear NF-κB localization were scored in at least 1500 cells for condition. Means ± SEM of six independent experiments. ANOVA, Tukey post-hoc test, *P < 0.05, **P < 0.01, ***P < 0.001.
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CONFLICT OF INTERESTS
The authors have no competing interests to declare.

APPENDIX A. SUPPLEMENTARY DATA
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.08.005.

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