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

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**ABSTRACT**

**Objective:** Non-coding RNAs constitute a major fraction of the β-cell transcriptome. While the involvement of microRNAs is well established, the contribution of long non-coding RNAs (lncRNAs) in the regulation of β-cell functions and in diabetes development remains poorly understood. The aim of this study was to identify novel islet lncRNAs differently expressed in type 2 diabetes models and to investigate their role in β-cell failure and in the development of the disease.

**Methods:** Novel transcripts dysregulated in the islets of diet-induced obese mice were identified by high throughput RNA-sequencing coupled with de novo annotation. Changes in the level of the lncRNAs were assessed by real-time PCR. The functional role of the selected lncRNAs was determined by modifying their expression in MIN6 cells and primary islet cells.

**Results:** We identified about 1500 novel lncRNAs, a number of which were differentially expressed in obese mice. The expression of two lncRNAs highly enriched in β-cells, linc2 and linc3, correlated to body weight gain and glycemia levels in obese mice and was also modified in diabetic db/db mice. The expression of both lncRNAs was also modulated in vitro in isolated islet cells by glucolipotoxic conditions. Moreover, the expression of the human orthologue of linc3 was altered in the islets of type 2 diabetic patients and was associated to the BMI of the donors. Modulation of the level of linc2 and linc3 by overexpression or downregulation in MIN6 and mouse islet cells did not affect insulin secretion but increased β-cell apoptosis.

**Conclusions:** Taken together, the data show that lncRNAs are modulated in a model of obesity-associated type 2 diabetes and that variations in the expression of some of them may contribute to β-cell failure during the development of the disease.

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**Keywords** Diabetes; Insulin; Pancreatic islet; Obesity; Gene expression

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, IncRNAs 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 IncRNAs have

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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, leptomycin 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 (BioServ F-3282, 60% energy from fat, Frenchtown, 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 florescence protein in β-cells (RIPPY) were obtained by crossing rosa-26EYFP 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 RIPPY 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/Center (University of Oulu) through the Nordic Network for Islet Transplantation, Uppsala University. After isolation, the human islets were utilized for RNA isolation or cultured in CMLR 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 mice and human islets preparation was performed 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 Ribo-Zero Magnetic 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 the lncRNA 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 ≥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 ≤0.05 were considered differentially expressed. Differential analysis by transcripts was done using Cuffdiff, v2.1.1 [28], on a gff file containing the coordinates of the novel transcripts. Gene ontology analysis was performed by submitting the gene 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 iTQ SYBR 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 Qiazol 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 x 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 DNAsse I for 30 min at 4 °C. The samples were then centrifuged at 17,000 x g for 20 min and the pellet collected as nucleoplastic 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 IncRNA 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 IncRNA expression and different characteristics of the individuals were performed by linear regression, where F-test was used to determine significance at p < 0.05.

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

3. RESULTS
To investigate the contribution of lncRNAs 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 1588 were novel. We compared our mouse data with those of Moran et al. [34] obtained in human using TransMap, 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 lncRNAs overlapped that of UCSC-annotated lncRNAs 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 lncRNAs 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 lncRNAs are shown in Figure 1B. Since there is not yet a consensus for the nomenclature of mouse IncRNAs, they are hereafter referred to as linc2 and linc3 (linc3 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 linc2 and linc3 are provided in Figure 1E–F and in the Supplemental Table 8. Subcellular fractionation of MIN6B1 cells revealed that linc2 is present both in the cytosolic and in the nuclear fractions while linc3 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 (HDF), 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 linc2 was not increased in the LDR group with no glycemic alterations but was upregulated 49 times in the HDF group that shows mild hyperglycemia [31]. The expression of linc3 tended to decrease already in the LDR group but reached significance only in the HDF 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 linc2 was more pronounced than in HDF mice, with an up-regulation of 1802-fold, whereas the decrease in linc3 expression was similar to that observed in response to high-fat diet HDF group (Figure 2C–D). We then tested whether the expression of these two lncRNAs 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 linc2 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 linc2 was mainly observed in animals weighing >40 g, suggesting the existence of a threshold. In contrast, the expression of linc3 was negatively correlated with these parameters except for insulinemia (Figure 3B,D,F).

To assess whether the expression of these two novel lncRNAs is restricted to pancreatic islets, we analyzed their levels in a large panel of tissues except in heart, where the expression is about 100 times lower compared to islets (Figure 4A). linc3 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 IncRNAs 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 IncRNAs. E. Locus architecture and isoforms of the linc2 gene. F. Locus architecture and isoforms of the linc3 gene.
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 IncRNA 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 IncRNAs. We mapped the identified mouse IncRNA sequences to the human genome using TransMap. To validate the predicted orthologues we designed primers in the putative exons. This enabled us to detect the orthologue for linc2 in the human genome using TransMap. To validate the predicted orthologues we designed primers in the putative exons. This enabled us to detect the orthologue for linc2 (region shown in Supplemental Table 10) but not for linc3. As was the case for its mouse orthologue, the expression of human LINC3 was not modified in the presence of 20 mM glucose but was decreased about 30% upon incubation with 0.5 mM palmitate (Figure 6).

In view of these findings, we assessed whether the expression of human LINC3 is diminished in the islets of T2D patients. The characteristics of the non-diabetic and diabetic donors are summarized in Figure 7A. Human LINC3 expression was 75% lower in donors with T2D than in controls (Figure 7B). Furthermore, as was the case in mice, there was a negative correlation between the level of this lncRNA and the BMI of the subjects (Figure 7C). We also observed a trend for a negative correlation between the level of this lncRNA and Hba1c, but the values did not reach statistical significance (Figure 7D). There was no correlation with the age of the donors (data not shown).

To assess the possible contribution of linc2 and linc3 to the regulation 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 IncRNAs 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 [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 IncRNAs. We found that incubation of the cells with leptin, 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

**Figure 2:** The expression levels of two IncRNAs are modified 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/+ , 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 IncRNAs 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/+.
overexpressing \( \beta \text{linc}2 \) alone, but, in the presence of leptomycin B, we observed an increase in the number of cells in which 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 IncRNA 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 IncRNAs 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 4: βlinc2 and βlinc3 are expressed in β-cells and their expression is higher in pancreatic islets compared to other tissues. A) Expression of βlinc2 and B) of βlinc3 in pancreatic islets and in different other tissues of C57BL/6 mice at 13 weeks of age. C) Expression of βlinc2 and D) of βlinc3 in whole islets and in FAC-sorted cells isolated from RIPYY mice. The expression levels were measured by real-time PCR and were normalized to the level detected in islets. T-test or ANOVA, Tukey post-hoc test. *P < 0.05 **P < 0.01 vs control (whole islets).
**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 IncRNAs 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 IncRNAs 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 \( \text{NF-\kappa B} \) nuclear translocation. Although basal \( \text{NF-\kappa B} \) activity is required for normal insulin release [47], its prolonged activation plays a central role in cytokine and in glucolipotoxic-induced \( \beta \)-cell death [37,40]. One of the initial events leading to the activation of \( \text{NF-\kappa B} \), 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 \( \text{NF-\kappa B} \) 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 \( \text{NF-\kappa B} \) 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 IncRNA on \( \text{NF-\kappa B} \) 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 \( \text{NF-\kappa B} \). 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 IncRNAs can affect the survival of \( \beta \)-cells. The IncRNAs Lethe and Nkila have been shown to interact directly with components of the NF-\kappaB pathway [50,51]. Future studies should determine whether this is also the case for \( \beta \text{linc2} \).
the expression of these transcripts was manipulated in vivo in mice \cite{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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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.

REFERENCES


