

linc1 encodes a long noncoding RNA that regulates islet -cell formation and function

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RESEARCH COMMUNICATION

***βlinc1* encodes a long noncoding RNA that regulates islet β-cell formation and function**Luis Arnes,¹ Ildem Akerman,^{2,3} Dina A. Balderes,¹ Jorge Ferrer,^{2,3} and Lori Sussel¹¹Department of Genetics and Development, Columbia University, New York, New York 10032, USA; ²Genomic Programming of B-Cells Laboratory, Institut d'Investigacions August Pi I Sunyer (IDIBAPS), Barcelona 08036, Spain;³Department of Medicine, Imperial College London, London W12 0NN, United Kingdom

Pancreatic β cells are responsible for maintaining glucose homeostasis; their absence or malfunction results in diabetes mellitus. Although there is evidence that long noncoding RNAs (lncRNAs) play important roles in development and disease, none have been investigated in vivo in the context of pancreas development. In this study, we demonstrate that *βlinc1* (β-cell long intergenic non-coding RNA 1), a conserved lncRNA, is necessary for the specification and function of insulin-producing β cells through the coordinated regulation of a number of islet-specific transcription factors located in the genomic vicinity of *βlinc1*. Furthermore, deletion of *βlinc1* results in defective islet development and disruption of glucose homeostasis in adult mice.

Supplemental material is available for this article.

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Type 2 diabetes (T2D) arises when pancreatic islets are no longer able to compensate for the increasing demand of insulin to maintain glucose homeostatic conditions. Despite the improvement in current therapies, >300 million people live with T2D, and those numbers are expected to almost double in the next 20 years. Accordingly, it is necessary to increase our understanding of the underlying biology of islet dysfunctions associated with T2D and identify novel druggable targets.

Studies in pancreas development have identified numerous transcription factors and lineage-specific regulatory networks that are required for the specification of the different pancreatic cell types, including acini, ducts, and endocrine cells (Arda et al. 2013). This functionally diverse group of cells arises from a common pool of pancreatic progenitors in two major waves of differentiation termed the primary and secondary transitions. Differentiation of the pancreatic β cells occurs during the secondary transition, and the signaling pathways and regulatory factors involved in this important developmental process have been well characterized (for review, see Pan and

Wright 2011). These studies have provided the platform to generate functional β cells in vitro for regenerative medicine and suggested novel therapeutic approaches to prevent and treat β-cell dysfunction.

The study of β-cell biology has been recently complemented by genome-wide transcriptome analysis and characterization of epigenetic modifications, suggesting that non-protein-coding regions of the genome are integral to the transcriptional network regulating development and function of β cells (for review, see Arnes and Sussel 2015). Furthermore, a large number of T2D-associated common variants that were identified in genome-wide association studies map to noncoding genomic regions (Pasquali et al. 2014). Although there is increasing evidence that long noncoding RNAs (lncRNAs) play an important role in development and disease (Batista and Chang 2013), none have been investigated in vivo in the context of pancreas development and β-cell function. In this study, we describe the functional characterization of a newly identified conserved lncRNA termed *βlinc1* (β-cell long intergenic noncoding RNA 1). We demonstrated that *βlinc1* is required for the proper specification and function of endocrine cells through the coordinated regulation of a number of islet-specific transcription factors located in the genomic vicinity of *βlinc1*. Furthermore, deletion of *βlinc1* results in defective islet development and disrupted glucose homeostasis in the adult. These results have important implications for the identification of novel regulatory mechanisms underlying T2D susceptibility and suggest that ncRNAs could represent novel therapeutic targets for the treatment of diabetes.

Results and Discussion*Characterization of βLINC1*

lncRNAs expressed in the pancreatic islet are often highly tissue-specific, associated with clusters of open chromatin, and located in the genomic vicinity of transcription factors involved in β-cell development and/or function (Ku et al. 2012; Moran et al. 2012). We analyzed *βLINC1* (formally HI-LNC15), a 6.8-kb post-transcriptionally processed human islet-specific transcript without coding potential (Moran et al. 2012) that is located in a region of open chromatin ~20 kb upstream of *NKX2.2*, an essential islet homeobox transcription factor gene (Supplemental Fig. 1; Sussel et al. 1998). *βLINC1* resides in a large syntenic block located on chromosome 20 in humans and chromosome 2 in mice (Fig. 1A). It is conserved in mammals, with the largest stretches of homology located at the putative promoter region, similar to many characterized lncRNAs (Carninci et al. 2005). This high level of conservation enabled us to identify an orthologous *βlinc1* transcript in mice (Fig. 1B). Mouse *βlinc1* is also predicted to have no coding potential (CPC score -0.261; CPAT 0.055) (Supplemental Fig. 2a), and comparative sequence analysis between the mouse and human *βlinc1* transcripts

[Keywords: β cell; long noncoding RNA; pancreas development]

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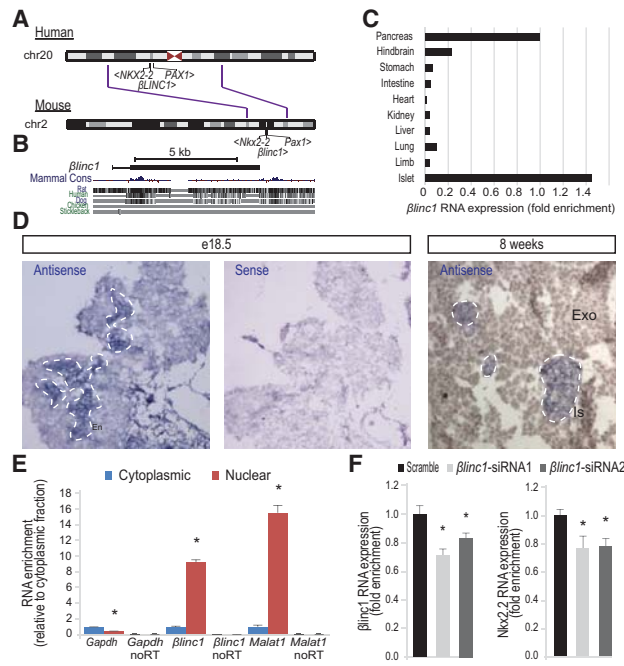


Figure 1. *βlinc1* is a conserved endocrine-specific lncRNA. (A) *βlinc1* is located in a large syntenic block on human chromosome 20 and mouse chromosome 2 (purple lines). The position and direction of *βlinc1* and the nearest adjacent genes are indicated. (B) Snapshot depicting *βlinc1* transcript structure generated by de novo assembly of RNA sequencing (RNA-seq) data from mouse embryonic day 14.5 (E14.5) pancreas and islet samples and 30-way Multiz Alignment and Conservation. The mouse *βlinc1* locus spans 8 kb, located in a gene desert between *Nkx2.2* and *Pax1* on the long (q) arm of chromosome 2 (chr2: 147,030,314–147,038,352, mm9), with a 73.6% sequence conservation with the human *βLINC1* locus as determined by LiftOver. (C) *βlinc1* RNA expression was determined by quantitative RT–PCR (qRT–PCR) in a tissue panel isolated from E15.5 embryos and adult islets. (D) RNA in situ hybridization of *βlinc1* in pancreatic sections of E18.5 embryos and adult pancreata showing enrichment of the *βlinc1* transcript in the trunk endocrine compartment and adult islets. White dotted lines depict the endocrine area and islets. The image is representative of at least three experiments. (E) Cellular fractionation of MIN6 cells showing that *βlinc1* is highly enriched in nuclear versus cytosolic fractions. Gapdh and Malat1 were included as negative and positive controls of nuclear transcript retention, respectively. Samples without the addition of reverse transcriptase (noRT) were included to control for genomic contamination. $n = 4$. (F) *βlinc1* and *Nkx2.2* expression in MIN6 cells treated with two different siRNAs against *βlinc1*. $n = 4$. Error bars represent \pm SEM. (*) $P < 0.05$, Student's *t*-test.

did not reveal any conserved small ORFs. In mouse islets, the genomic locus surrounding *βlinc1* is enriched in H3K4me1/3 and H3K27ac marks (Supplemental Fig. 2b), and there is evidence for NeuroD1, Pdx1, and Foxa2 binding at the putative *βlinc1* promoter region (Khoo et al. 2012; Jia et al. 2015). Despite these features, this 4.2-kb region of DNA within the *βlinc1* locus did not confer enhancer activity in luciferase reporter assays in MIN6 cells (Supplemental Fig. 3).

Expression studies revealed that *βlinc1* is enriched in embryonic pancreata and adult islets (Fig. 1C). RNA in situ analysis confirmed that *βlinc1* expression is restricted to adult islets and the trunk region of the developing pancreas (Fig. 1D). Furthermore, assessment of *βlinc1* expression in several islet cell lines demonstrated that *βlinc1* is enriched in insulin-producing cells (Supplemental Fig. 4a),

corresponding to its expression in FACS-purified human β cells (Supplemental Fig. 1).

βlinc1 RNA is retained in the nuclear fraction of β cells, further suggesting a role for *βlinc1* in transcriptional regulation (Fig. 1E). Although *βlinc1* is expressed at relatively low levels, the half-life of the *βlinc1* transcript is equivalent to that of *Tbp* (Supplemental Fig. 4b,c). This suggests that low transcript levels are not due to the degradation of aberrant transcripts, similar to what has been documented for other low-expressing lncRNAs (Clark et al. 2012).

Although there are limited tools available to predict the function of lncRNAs based on nucleotide sequence or genomic location, there is growing evidence that a subset of nuclear lncRNAs functions locally to regulate neighboring genes (Sauvageau et al. 2013; Vance et al. 2014). Consistently, siRNA-mediated knockdown of *βlinc1* RNA in MIN6 cells resulted in the down-regulation of the adjacent coding gene *Nkx2.2* (Fig. 1F), suggesting that the *βlinc1* transcript positively regulates the expression of *Nkx2.2*.

βlinc1 knockout mice are glucose-intolerant

To determine the in vivo function of *βlinc1*, we generated *βlinc1*-null mice (Supplemental Fig. 5). *βlinc1*^{+/-} and *βlinc1*^{-/-} mice were viable and fertile. Consistent with the expression of *βlinc1* being restricted to the developing endocrine pancreas and adult islet, *βlinc1*^{-/-} mice were mildly glucose-intolerant compared with *βlinc1*^{+/-} or *βlinc1*^{+/+} littermate controls (Fig. 2A). Furthermore, *βlinc1*^{-/-} mice displayed abnormal fasting plasma insulin levels and increased insulin secretion under low-glucose conditions and failed to elevate circulating insulin in response to glucose stimulation (Fig. 2B; Supplemental Fig. 6a). Interestingly, *βlinc1*^{-/-} mice were also mildly insulin-resistant despite the absence of *βlinc1* expression in muscle and subcutaneous white adipose tissue (Supplemental Fig. 6b,c). We did not detect additional phenotypes in *βlinc1*^{-/-} mice, particularly in other tissues where *Nkx2.2* is expressed, such as the brain and intestine, which is also consistent with the restricted expression and function of the *βlinc1* transcript to the endocrine pancreas.

Endocrine development is affected in *βlinc1*^{-/-} mice

Given the expression of *βlinc1* in the embryonic pancreas, it was possible that aberrant glucose homeostasis was caused by defects in pancreas development. Perinatal *βlinc1*^{-/-} mice did not exhibit overt defects in pancreas morphology but displayed a 50% reduction in β cells and an increase in the number of somatostatin-expressing cells (Supplemental Fig. 7a,b; data not shown). We did not detect alterations in islet cell ratios in *βlinc1*^{+/-} neonates (data not shown). The changes in islet cell ratios in the *βlinc1*^{-/-} animals appeared to be initiated during development at the secondary transition. At embryonic day 15.5 (E15.5), the number of insulin-producing β cells was already significantly reduced, and there was an approximately threefold increase in the number of somatostatin-producing cells (Fig. 2C,D; Supplemental Fig. 7c). There was also a smaller but significant reduction in the number of glucagon- and ghrelin-producing cells (Supplemental Fig. 7d–g). Changes in the expression of the corresponding hormone genes and β -cell-specific transcription

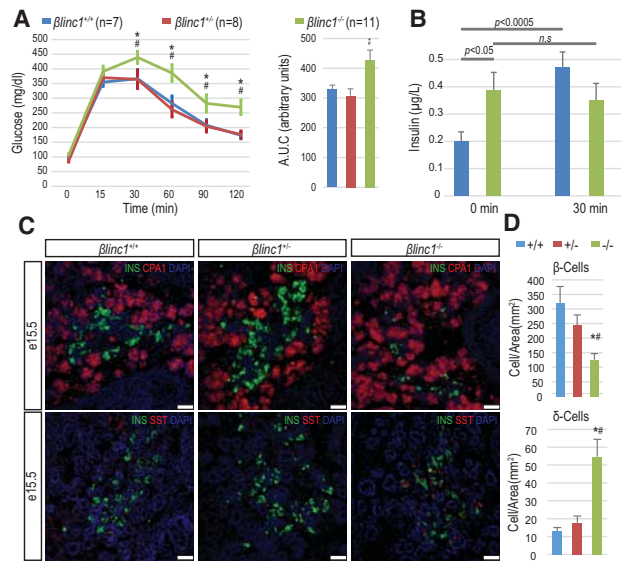


Figure 2. $\beta linc1$ knockout mice are glucose-intolerant and have defects in endocrine specification. (A) $\beta linc1^{-/-}$ 16-wk-old mice are mildly glucose-intolerant. (AUC) Area under the curve. (B) Plasma insulin levels in 16-wk-old $\beta linc1^{-/-}$ mice (green) ($n = 10$) and littermate $\beta linc1^{+/+}$ control mice (blue) ($n = 7$) at 0 and 30 min after glucose injection. (C) Immunofluorescence analysis of pancreas development at E15.5 showing a reduction of β cells and an increase of somatostatin-producing δ cells in $\beta linc1^{-/-}$ mice compared with $\beta linc1^{+/+}$ and $\beta linc1^{+/-}$ mice. There are no apparent morphological defects in the exocrine compartment, which was visualized by immunostaining against CPA1. Images are representative of $n > 3$ mice. Bar, 50 μ m. (D) Quantification of hormone-producing cells in E15.5 pancreata. $n = 4$. Error bars represent \pm SEM. (*) $P < 0.05$ $\beta linc1^{-/-}$ versus $\beta linc1^{+/+}$; (#) $P < 0.05$ $\beta linc1^{-/-}$ versus $\beta linc1^{+/-}$, Student's t -test.

factors were consistent with the changes in cell numbers (Fig. 3A,B). Interestingly, we did not observe up-regulation of δ -cell markers, such as *Hhex* and *Crhr2*, suggesting that deletion of $\beta linc1$ may not cause the formation of excess bona fide δ cells. However, we also did not detect co-expression of SST with the other endocrine hormones (Supplemental Fig. 8), indicating that the increased numbers of SST-expressing cells may arise from precocious or de novo differentiation of immature and/or noncanonical δ -cell populations.

The altered changes in islet cell ratios in $\beta linc1^{-/-}$ embryos also did not appear to be caused by aberrant proliferation or apoptosis, since there were no detectable differences in pHistone H3 or cleaved caspase 3 staining between the mutant and wild-type embryos (data not shown). In support of a role for $\beta linc1$ in the proper specification of some endocrine cell lineages, *Neurog3* is also decreased (Fig. 3B). Importantly, there was no effect on expression of the exocrine marker *Ptf1a* (Fig. 3B), confirming the endocrine specificity of $\beta linc1$ function.

$\beta linc1$ deficiency affects the expression of several genes related to endocrine cell differentiation and β -cell function

To identify the pathways regulated by $\beta linc1$, we performed global transcriptome analysis on E15.5 $\beta linc1^{-/-}$ versus $\beta linc1^{+/+}$ pancreata (Gene Expression Omnibus [GEO] accession no. GSE73711) (Supplemental Table 1).

Consistent with the changes in endocrine cell ratios, these data indicated a general dysregulation of endocrine system development in the absence of $\beta linc1$ (Supplemental Fig. 9a,b). Notably, we did not detect changes in genes coding for transcription factors involved in the specification and maintenance of pancreas progenitors; however, in the absence of $\beta linc1$, a large number of genes involved in the specification of endocrine progenitors and in the maturation and function of β cells were dysregulated (Supplemental Fig. 9c). This supports a role for $\beta linc1$ in the proper specification of endocrine progenitors during the secondary transition of pancreas development, when the majority of β -cell differentiation occurs.

Although we did not detect altered islet cell ratios or glucose metabolism defects in the heterozygous $\beta linc1^{+/-}$ pancreata, (Fig. 2a; Supplemental Fig. 7), $\beta linc1^{+/-}$ embryos had reduced $\beta linc1$ transcript and displayed defects in the expression of several islet transcription factors, including *Nkx2.2*, *Pax6*, and *Mafb* (Fig. 3B). To identify additional gene expression changes that were not confounded by a change in islet cell type ratios, we performed transcriptome analysis on E15.5 $\beta linc1^{+/-}$ pancreata. Strikingly, there were many genes dysregulated in pancreata lacking one copy of $\beta linc1$ (Fig. 4A; Supplemental Table 1). This analysis also revealed that, in addition to *Nkx2.2*, *Pax6*, and *Mafb*, five of the top 10 most significantly dysregulated genes in the $\beta linc1^{+/-}$ mice were also located within an ~ 55 -Mb region on chromosome 2 (Fig. 4B,C). qPCR analysis verified that these five down-regulated genes on chromosome 2 were significantly decreased in both $\beta linc1^{+/-}$ and $\beta linc1^{-/-}$ pancreata (Fig. 4D).

Dysregulated genes in $\beta linc1^{+/-}$ localize to the genomic vicinity of $\beta linc1$

To determine whether the genes regulated by $\beta linc1$ were biased based on their genomic location, we positionally mapped all genes that were significantly dysregulated ($P < 0.05$) in $\beta linc1^{+/-}$ pancreata versus the wild-type controls. Remarkably, a disproportionate number of dysregulated genes mapped to a discrete region of chromosome 2, within 40 Mb of the $\beta linc1$ locus. Importantly, this is not due to an increased number of genes that are expressed on chromosome 2 during pancreas development (Supplemental Fig. 10a,b). Moreover, gene ontology analysis

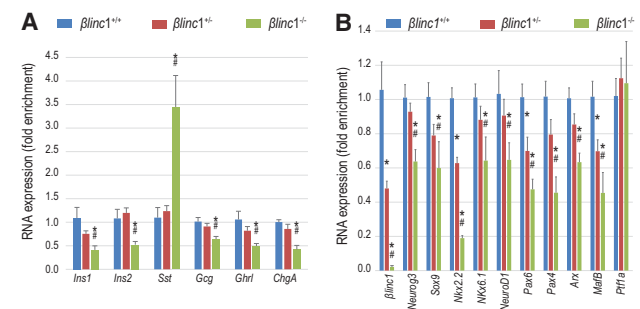


Figure 3. $\beta linc1$ regulates the expression of endocrine-specific genes. (A) qPCR analysis of hormones in E15.5 pancreata. General reduction of hormone expression with the up-regulation of somatostatin. $n \geq 5$. (B) qPCR analysis of $\beta linc1$ and several transcription factors involved in pancreas development in E15.5 pancreata. $n \geq 5$. Error bars represent \pm SEM. (*) $P < 0.05$ $\beta linc1^{-/-}$ versus $\beta linc1^{+/+}$; (#) $P < 0.05$ $\beta linc1^{+/-}$ versus $\beta linc1^{+/+}$, Student's t -test.

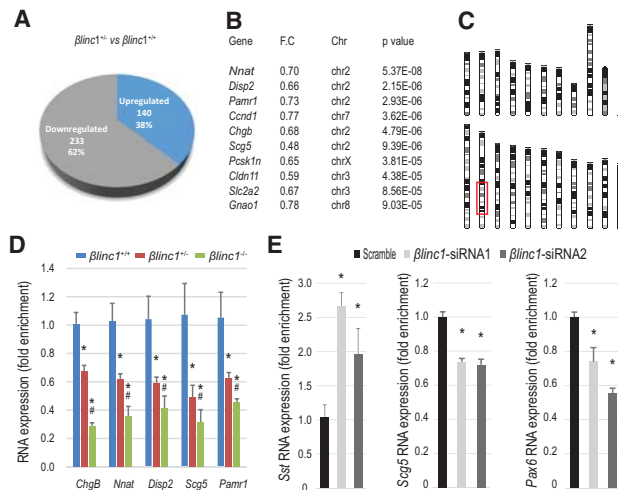


Figure 4. *βlinc1* predominantly regulates the expression of β-cell-enriched genes that map to the same chromosomal region as *βlinc1*. (A) Pie chart showing the total numbers and relative percentages of up-regulated and down-regulated genes ($P < 0.05$) in *βlinc1*^{-/-} versus *βlinc1*^{+/+}. (B) Fold change and chromosomal location of the 10 most significantly down-regulated genes in *βlinc1*^{-/-} compared with *βlinc1*^{+/+}. A large proportion of these genes is in chromosome 2 (Supplemental Fig. 10a). (C) Schematic representation of the mouse karyotype. The red box denotes the location of the dysregulated genes on chromosome 2 in *βlinc1*^{-/-} compared with *βlinc1*^{+/+} mice. (D) qRT-PCR validation of RNA-seq results for the genes located in chromosome 2. (*) $P < 0.05$ versus *βlinc1*^{+/+}; (#) $P < 0.05$ versus *βlinc1*^{+/+}. (E) Down-regulation of *βlinc1* with two different siRNAs in MIN6 cells recapitulates the increase in somatostatin expression and down-regulation of *Scg5* and *Pax6* seen in vivo. $n = 4$. Error bars represent \pm SEM. (*) $P < 0.05$ versus scrambled. Student's *t*-test.

showed that the dysregulated genes on chromosome 2 are associated with endocrine development and islet morphology. Ingenuity Pathway Analysis also indicated that the predominant upstream regulators affected by loss of one allele of *βlinc1* are the known islet-specific transcription factors *Pdx1* and *NeuroD1*. These pancreas-related categories are lost if the same analysis is performed with all of the genes that are located in this region of chromosome 2 (Supplemental Fig. 10a,b).

The remarkable observation that many *βlinc1*-regulated genes were located on the same chromosome as *βlinc1* prompted us to investigate whether the observed gene expression changes were due to loss of *βlinc1* transcript rather than the deletion of a DNA enhancer element, similar to what was observed with *Nkx2.2* regulation (Fig. 1F). Assessment of the expression of additional candidate *βlinc1* target genes located on chromosome 2 in response to siRNA-mediated knockdown of *βlinc1* in MIN6 cells revealed the up-regulation of *Sst* expression and down-regulation of *Scg5* and *Pax6*, confirming the biological role of the *βlinc1* transcript in gene regulation (Fig. 4E). Moreover, shRNA-mediated knockdown of the orthologous human *βLINC1* transcript in the human insulin-producing EndoC-βH1 cell line also causes altered expression of genes dysregulated in *βlinc1*^{-/-} mice (I Akerman and J Ferrer, in prep.). Therefore, it appears that the *βlinc1* transcript regulates the expression of a set of functionally related genes that are important for the development and/or function of β cells.

In this study, we describe the first in vivo characterization of a conserved islet-specific lncRNA. Homozygous

deletion of *βlinc1* in mice causes the down-regulation of important islet transcription factors and impaired β-cell specification during embryonic development. Furthermore, adult mice lacking *βlinc1* are glucose-intolerant and display defective insulin secretion. Although it is possible that a subset of the observed *βlinc1*^{-/-} phenotypes could be due to the deletion of a regulatory element within the genomic locus, siRNA- and shRNA-mediated knockdown experiments of the *βlinc1*RNA in mouse MIN6 cells (Figs. 1F, 4E) and human EndoC-βH1 cells recapitulated some of the gene changes observed in vivo. Furthermore, the *βlinc1* genomic locus does not appear to have enhancer activities in MIN6 cells (Supplemental Fig. 3); however, limitations of these in vitro studies prevented us from ascribing all of the *βlinc1* phenotypes to loss of the *βlinc1* transcript. Interestingly, although we demonstrated that the *βlinc1* transcript regulates several important islet transcription factors, only a subset of the *βlinc1*^{-/-} phenotypes could be attributed to reduction of *Nkx2.2*, *Pax6*, and/or *MafB* expression (Supplemental Table 2; Pan and Wright 2011), suggesting that *βlinc1* is involved in novel lineage regulatory pathways.

Transcriptome analysis of pancreata lacking one copy of *βlinc1* also revealed the dysregulation of a set of functionally related β-cell genes located in discrete genomic regions. It is particularly intriguing that *βlinc1* specifically regulates three essential islet transcription factors, *ChgB*, and additional β-cell genes on chromosome 2. Long-range interchromosomal and intrachromosomal interactions through chromosomal looping and transcription factories are known to be involved in the transcriptional coregulation of functionally related genes in a cell type-specific manner (Schoenfelder et al. 2010; Papantonis et al. 2012). For example, insulin expression and calcium signaling have been shown to be coupled through a long-range chromosomal interaction that spans >65 Mb (Xu et al. 2014). lncRNAs have also been implicated in higher-order gene regulation through chromatin organization, and there is evidence of lncRNAs being highly enriched at transcription “factories” (Caudron-Herger et al. 2015). Therefore, it is possible that *βlinc1* coordinately regulates β-cell gene expression through the structural organization of the chromatin in β cells. Analysis of the known chromatin features and topologically associated domains (TADs) in and around *βlinc1* failed to reveal any obvious domains or features that could explain the regulation of the genes on chromosome 2. HiC analyses suggested that *βlinc1* may be located at the edge of a TAD (Supplemental Fig. 11; Shen et al. 2012); however, the presence of this domain does not correlate with the observed in vivo gene expression changes. For example, the TAD data include all of the genes located between *Insm1* and *Foxa2*, but only two of the 13 genes within this domain (*Nkx2-2* and *Insm1*) are down-regulated in *βlinc1*^{-/-} mice.

Although we are just beginning to understand a role for lncRNAs in the cell, many findings have suggested that lncRNAs provide a layer of cell-specific gene regulation that contributes to cellular diversity (Mattick 2001). It is therefore intriguing that *βlinc1* has such a limited expression domain, and the phenotype of the global *βlinc1*^{-/-} mice is primarily restricted to β-cell formation and function. Furthermore, our discovery of *βlinc1* as a novel islet-specific transcriptional regulator has important implications for understanding β-cell biology and suggests that ncRNAs could represent novel therapeutic targets for the treatment of diabetes.

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Materials and methods

Generation of the *blinc1* knockout allele

The *blinc1* knockout allele was generated using the Recombinase Mediate Cassette Exchange protocol as previously described (Arnes et al. 2012) with some modifications. Short arms (≈ 500 base pairs) homologous to the flanking region of *blinc1* and to a downstream region (2.9 kb) were generated by PCR and cloned into pLCA.71/2272 and pMCS-DTA. The BAC clone (RP23-236P19) was modified in two consecutive recombineering steps in SW106 cells: The *blinc1* sequence was replaced with the *pu Δ tk-EM7-kan* cassette, and the DTA-Amp^r cassette was inserted 2.9 kb downstream from *blinc1*, resulting in the replacement of 1 kb of genomic DNA (Supplemental Fig. 5b). Positive clones were validated by PCR and DNA sequencing, and a correctly modified BAC was electroporated into mouse embryonic stem cells at Columbia University (Herbert Irving Comprehensive Cancer Center Transgenic Shared Resource). Potentially recombined clones were screened by PCR with primers P1 and P2 (Supplemental Fig. 5d,e). Two positive clones were used to generate chimeric mice that resulted in germline transmission.

Mice

All mice were maintained on a mixed C57BL/6J129SV genetic background. All animals were maintained according to Columbia University Institutional Animal Care and Use Committee approval protocol AAAG3206.

Physiological assays

Glucose tolerance tests, measurement of plasma insulin, and insulin secretion assays were performed as previously described (Gu et al. 2010).

Cell lines and transfection

MIN6, α TC1 clone 6 (American Type Culture Collection), and PG1 cells (provided by Dr. Jeffrey Zigman, University of Texas Southwestern Medical Center) were passaged and maintained following standard techniques in 5% CO₂ and 95% air. MIN6 cells were transfected with 10 nM siRNA targeting *blinc1* and a scrambled control (Silencer Select, Ambion) using Lipofectamine 2000 following the manufacturer's instructions (Life Technologies). siRNAs targeting *blinc1* were designed using the algorithm provided by the manufacturer, and sequences are listed in Supplemental Table 3.

Enhancer activity

A 4.2-kb fragment of the *blinc1* genomic locus (chr2: 147,030,443–147,034,638, mm9) was cloned into the PGL4.27 luciferase vector. One microgram of the experimental vector PGL4.27-*blinc1* region and the positive controls (CDKN2BAS enhancer, Ins2 promoter, and NeuroD promoter/enhancer) were individually cotransfected with 0.1 μ g of pRL into MIN6 cells in triplicate. Luciferase activity was measured after 48 h. PGL4.23-CDKN2BAS was a gift from Jorge Ferrer (Addgene plasmid no. 60296). PGL3-Ins2 and PGL3-NeuroD have been previously described (Raum et al. 2006; Anderson et al. 2009). Luciferase values were normalized to Renilla activity to account for transfection efficiencies and were expressed as fold increase over the empty vector.

RNA in situ hybridization

The *blinc1* probe was generated by PCR from E15.5 pancreas cDNA and cloned into pCRII-TOPO (Life Technologies). Sense and antisense probes were labeled with the DIG RNA-labeling mix (Roche Applied Science). RNA in situ hybridization was performed as previously described (Mastracci et al. 2013), including a short treatment of proteinase K digestion.

Immunofluorescence

Tissue processing and immunofluorescence analysis were performed as previously described (Arnes et al. 2012). Primary antibodies are listed in

Supplemental Table 3. DAPI (1:1000; Invitrogen) was applied for 30 min following secondary antibody incubation: DyLight-488, DyLight-549, DyLight-649, Alexa-488, and Alexa-647 (Jackson ImmunoResearch). Images were acquired with either an epifluorescence (Leica DM5500) or a confocal (Zeiss LSM 710) microscope.

Morphometric analysis and cell counting

Morphometric analysis was performed in E15.5 and postnatal day 2 pancreata. The entire organ was sectioned, and at least six evenly distributed sections were analyzed. The number of endocrine cells was determined relative to the total pancreatic area. Pancreas area was quantified using Image Pro Plus 5.0.1 software (Media Cybernetics).

Cellular fractionation

MIN6 cells were grown to confluency, detached by trypsinization, and pelleted. Half of the pellet was used for total RNA isolation, and the other half was used for nuclear and cytoplasmic isolation using the PARIS kit (Ambion) following the manufacturer's instructions.

RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated and analyzed as previously described (Arnes et al. 2012). Primers and probes are listed in Supplemental Table 3.

RNA sequencing (RNA-seq) analysis

Total RNA from *blinc1*^{+/+}, *blinc1*^{+/-}, and *blinc1*^{-/-} mice was converted into cDNA libraries (TruSeq RNA sample preparation kit version 2, Illumina) using poly-A pull-down for mRNA enrichment. Sequencing was performed to a depth of 30 million pairs in three biological replicates per condition. Differential expression between replicates was assessed using DESeq (R package). All samples had RNA integrity (RIN) values >9.0 as determined with Agilent Bioanalyzer 2100. Complete RNA-seq data are available through GEO accession number GSE73711.

TADs

TADs in the genomic vicinity of *blinc1* were identified from the mouse encode project at the Ren laboratory (<http://chromosome.sdsc.edu/mouse>).

Statistics

All data were expressed as mean \pm SEM. Mean and SEM values were calculated from at least three biological replicates. The statistical significance was measured by two-tailed Student's *t*-test.

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References

- Anderson KR, Torres CA, Solomon K, Becker TC, Newgard CB, Wright CV, Hagman J, Sussel L. 2009. Cooperative transcriptional regulation of the essential pancreatic islet gene *NeuroD1* ($\beta 2$) by *Nkx2.2* and *neurogenin 3*. *J Biol Chem* **284**: 31236–31248.
- Arda HE, Benitez CM, Kim SK. 2013. Gene regulatory networks governing pancreas development. *Dev Cell* **25**: 5–13.
- Arnes L, Sussel L. 2015. Epigenetic modifications and long noncoding RNAs influence pancreas development and function. *Trends Genet* **31**: 290–299.
- Arnes L, Hill JT, Gross S, Magnuson MA, Sussel L. 2012. Ghrelin expression in the mouse pancreas defines a unique multipotent progenitor population. *PLoS One* **7**: e52026.
- Batista PJ, Chang HY. 2013. Long noncoding RNAs: cellular address codes in development and disease. *Cell* **152**: 1298–1307.
- Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, Oyama R, Ravasi T, Lenhard B, Wells C, et al. 2005. The transcriptional landscape of the mammalian genome. *Science* **309**: 1559–1563.
- Caudron-Herger M, Cook PR, Rippe K, Papantonis A. 2015. Dissecting the nascent human transcriptome by analysing the RNA content of transcription factories. *Nucleic Acids Res* **43**: e95.
- Clark MB, Johnston RL, Inostroza-Ponta M, Fox AH, Fortini E, Moscato P, Dinger ME, Mattick JS. 2012. Genome-wide analysis of long noncoding RNA stability. *Genome Res* **22**: 885–898.
- Gu C, Stein GH, Pan N, Goebels S, Hornberg H, Nave KA, Herrera P, White P, Kaestner KH, Sussel L, et al. 2010. Pancreatic β cells require *NeuroD* to achieve and maintain functional maturity. *Cell Metab* **11**: 298–310.
- Jia S, Ivanov A, Blasevic D, Muller T, Purfurst B, Sun W, Chen W, Poy MN, Rajewsky N, Birchmeier C. 2015. *Insm1* cooperates with *Neurod1* and *Foxa2* to maintain mature pancreatic β -cell function. *EMBO J* **34**: 1417–1433.
- Khoo C, Yang J, Weinrott SA, Kaestner KH, Naji A, Schug J, Stoffers DA. 2012. Research resource: the *pdx1* cisrome of pancreatic islets. *Mol Endocrinol* **26**: 521–533.
- Ku GM, Kim H, Vaughn IW, Hangauer MJ, Myung Oh C, German MS, McManus MT. 2012. Research resource: RNA-seq reveals unique features of the pancreatic β -cell transcriptome. *Mol Endocrinol* **26**: 1783–1792.
- Mastracci TL, Lin CS, Sussel L. 2013. Generation of mice encoding a conditional allele of *Nkx2.2*. *Transgenic Res* **22**: 965–972.
- Mattick JS. 2001. Non-coding RNAs: the architects of eukaryotic complexity. *EMBO Rep* **2**: 986–991.
- Moran I, Akerman I, van de Bunt M, Xie R, Benazra M, Nammo T, Arnes L, Nakic N, Garcia-Hurtado J, Rodriguez-Segui S, et al. 2012. Human β cell transcriptome analysis uncovers lincRNAs that are tissue-specific, dynamically regulated, and abnormally expressed in type 2 diabetes. *Cell Metab* **16**: 435–448.
- Pan FC, Wright C. 2011. Pancreas organogenesis: from bud to plexus to gland. *Dev Dyn* **240**: 530–565.
- Papantonis A, Kohro T, Baboo S, Larkin JD, Deng B, Short P, Tsutsumi S, Taylor S, Kanki Y, Kobayashi M, et al. 2012. TNF α signals through specialized factories where responsive coding and miRNA genes are transcribed. *EMBO J* **31**: 4404–4414.
- Pasquali L, Gaulton KJ, Rodriguez-Segui SA, Mularoni L, Miguel-Escalada I, Akerman I, Tena JJ, Moran I, Gomez-Marin C, van de Bunt M, et al. 2014. Pancreatic islet enhancer clusters enriched in type 2 diabetes risk-associated variants. *Nat Genet* **46**: 136–143.
- Raum JC, Gerrish K, Artner I, Henderson E, Guo M, Sussel L, Schisler JC, Newgard CB, Stein R. 2006. *FoxA2*, *Nkx2.2*, and *PDX-1* regulate islet β -cell-specific *mafA* expression through conserved sequences located between base pairs –8118 and –7750 upstream from the transcription start site. *Mol Cell Biol* **26**: 5735–5743.
- Sauvageau M, Goff LA, Lodato S, Bonev B, Groff AF, Gerhardinger C, Sanchez-Gomez DB, Haciasuleyman E, Li E, Spence M, et al. 2013. Multiple knockout mouse models reveal lincRNAs are required for life and brain development. *Elife* **2**: e01749.
- Schoenfelder S, Sexton T, Chakalova L, Cope NF, Horton A, Andrews S, Kurukuti S, Mitchell JA, Umlauf D, Dimitrova DS, et al. 2010. Preferential associations between co-regulated genes reveal a transcriptional interactome in erythroid cells. *Nat Genet* **42**: 53–61.
- Shen Y, Yue F, McCleary DF, Ye Z, Edsall L, Kuan S, Wagner U, Dixon J, Lee L, Lobanenkov VV, et al. 2012. A map of the *cis*-regulatory sequences in the mouse genome. *Nature* **488**: 116–120.
- Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL, German MS. 1998. Mice lacking the homeodomain transcription factor *Nkx2.2* have diabetes due to arrested differentiation of pancreatic β cells. *Development* **125**: 2213–2221.
- Vance KW, Sansom SN, Lee S, Chalei V, Kong L, Cooper SE, Oliver PL, Ponting CP. 2014. The long non-coding RNA *Paupar* regulates the expression of both local and distal genes. *EMBO J* **33**: 296–311.
- Xu Z, Lefevre GM, Gavrilova O, Foster St Claire MB, Riddick G, Felsenfeld G. 2014. Mapping of long-range INS promoter interactions reveals a role for calcium-activated chloride channel ANO1 in insulin secretion. *Proc Natl Acad Sci* **111**: 16760–16765.



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