Cell Metabolism

Human Pancreatic β Cell IncRNAs Control Cell-Specific Regulatory Networks

Graphical Abstract

Highlights

- A loss-of-function screen reveals functional β cell IncRNAs
- Cell-specific IncRNAs and transcription factors regulate common gene networks
- The IncRNA PLUTO influences interactions between an enhancer cluster and PDX1
- PLUTO and PDX1 are deregulated in type 2 diabetes and impaired glucose tolerance

Authors

Ildem Akerman, Zhidong Tu, Anthony Beucher, ..., Eric Schadt, Philippe Ravassard, Jorge Ferrer

Correspondence
jferrerm@imperial.ac.uk

In Brief

Akerman et al. studied the function of human β cell IncRNAs with RNAi, CRISPRi, and co-expression networks. This revealed β cell IncRNAs and transcription factors that control common regulatory networks. One IncRNA, PLUTO, is downregulated in type 2 diabetes and controls PDX1, encoding a key β cell transcription factor.

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Human Pancreatic β Cell IncRNAs Control Cell-Specific Regulatory Networks

Ildem Akerman,1,2,3 Zhidong Tu,3 Anthony Beucher,1 Delphine M.Y. Rolando,1 Claire Sauty-Colace,5 Marion Benazra,5 Nikolina Nakic,1 Jialiang Yang,4 Huan Wang,4 Lorenzo Pasquali,3,6 Ignasi Moran,1 Javier Garcia-Hurtado,2,3 Natalia Castro,2,3 Roser Gonzalez-Franco,1 Andrew F. Stewart,7 Carmen Argmann,4 Eric Schadt,4 Philippe Ravassard,5 and Jorge Ferrer1,2,3,12,*

1Section of Epigenomics and Disease, Department of Medicine, Imperial College London, London W12 ONN, United Kingdom
2Genomic Programming of Beta Cells Laboratory, Instituto d’Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona 08036, Spain
3Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Madrid 28029, Spain
4Department of Genetics and Genomic Science, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
5Sorbonne Universités, UPMC Univ Paris 06, INSERM, CNRS, Institut du cerveau et de la moelle (ICM) – Hôpital Pitie-Salpêtrière, Boulevard de l’Hôpital, Paris 75013, France
6Germans Trias i Pujol University Hospital and Research Institute and Josep Carreras Leukaemia Research Institute, Badalona 08916, Spain
7Diabetes, Obesity, and Metabolism Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
8European Genomic Institute for Diabetes, INSERM UMR 1190, Lille 59800, France
9Diabetes Research Institute (HSR-DRI), San Raffaele Scientific Institute, Milano 20132, Italy
10Cell Isolation and Transplantation Center, University of Geneva, 1211 Geneva 4, Switzerland
11Department of Clinical Sciences, Lund University Diabetes Centre, Lund University, Lund 20502, Sweden
12Lead Contact
*Correspondence: jferrerim@imperial.ac.uk
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SUMMARY

Recent studies have uncovered thousands of long non-coding RNAs (IncRNAs) in human pancreatic β cells. β cell IncRNAs are often cell type specific and exhibit dynamic regulation during differentiation or upon changing glucose concentrations. Although these features hint at a role of IncRNAs in β cell gene regulation and diabetes, the function of β cell IncRNAs remains largely unknown. In this study, we investigated the function of β cell-specific IncRNAs and transcription factors using transcript knockdowns and co-expression network analysis. This revealed IncRNAs that function in concert with transcription factors to regulate β cell-specific transcription networks. We further demonstrate that the IncRNA PLUTO affects local 3D chromatin structure and transcription of PDX1, encoding a key β cell transcription factor, and that both PLUTO and PDX1 are downregulated in islets from donors with type 2 diabetes or impaired glucose tolerance. These results implicate IncRNAs in the regulation of β cell-specific transcription factor networks.

INTRODUCTION

Transcriptome surveys have uncovered tens of thousands of mammalian transcripts longer than 200 nucleotides that have low protein-coding potential (Carninci et al., 2005; Derrien et al., 2012; Guttmann et al., 2009). A small fraction of these long non-coding RNAs (IncRNAs) have been shown to control gene expression by modulating chromosomal structure, transcription, splicing, mRNA transport, stability, or translation (Carrilier et al., 2012; Chen and Carmichael, 2009; Gong and Maquat, 2011; Lai et al., 2013; Luco and Misteli, 2011; Willingham et al., 2005; Yao et al., 2010). Specific IncRNAs have thus been implicated in various key processes, including random X chromosome inactivation, imprinting, the cell cycle, organogenesis, differentiation, pluripotency, and cancer progression (Guttmann et al., 2011; Huarte et al., 2010; Hung et al., 2011; Klattenhoff et al., 2013; Kretz et al., 2013; Penny et al., 1996; Schmitt and Chang, 2013; Sweats et al., 2002; Uiltsky et al., 2011). Despite these wide-ranging biological roles, the fraction of IncRNAs that is genuinely functional and the true impact of IncRNAs in human biology and disease remain poorly understood.

Pancreatic β cells regulate glucose homeostasis by secreting insulin and play a central role in the pathogenesis of major forms of diabetes mellitus. Recently, more than 1,100 IncRNAs were identified in human pancreatic islets and purified β cells (Morán et al., 2012) as well as in mouse pancreatic islet cells (Benner et al., 2014; Ku et al., 2012; Morán et al., 2012). A large fraction of human β cell IncRNAs are cell-specific, and several are known to be activated during β cell differentiation (Morán et al., 2012). This cellular specificity has also been noted for IncRNAs in other cell types (Cabit et al., 2011; Derrien et al., 2012) and points to the possibility that IncRNAs may regulate genetic programs important for lineage-specific differentiation or specialized cellular functions. Further, several β cell IncRNAs were shown to be regulated by extracellular glucose concentrations, suggesting a potential role of IncRNAs in the functional adaptation of β cells to increased insulin secretory demands (Morán et al., 2012; Guttmann et al., 2009).
Some islet lncRNAs map to loci that contain polygenic or Mendelian defects associated with human diabetes, whereas selected lncRNAs show deregulation in islets from organ donors with human type 2 diabetes (T2D) (Fadista et al., 2014; Morán et al., 2012). Collectively, these properties define a newly identified class of candidate regulators of β cell differentiation and function, with potential implications for human diabetes mellitus. However, the true relevance of β cell lncRNAs depends on whether they elicit a physiological function in human β cells, which remains to be addressed systematically.

In the current study, we have focused on a set of lncRNAs that show restricted expression in human pancreatic β cells and have tested the hypothesis that they regulate β cell gene expression. Our studies have uncovered a regulatory network in which lineage-specific lncRNAs and transcription factors (TFs) control common genes. Furthermore, we show that lncRNAs frequently regulate genes associated with clusters of islet enhancers, which have previously been shown to be the primary functional targets of islet-specific TFs. We performed a detailed analysis of a specific lncRNA named PLUTO, which controls PDX1, a master regulator of pancreas development and β cell differentiation and, thereby, modulates the PDX1-dependent transcriptional program. Finally, we show that PLUTO and PDX1 are downregulated in islets from organ donors with type 2 diabetes or impaired glucose tolerance, suggesting a potential role in human diabetes.

RESULTS

Human β Cell lncRNA Knockdowns Cause Profound Transcriptional Phenotypes

To directly test the regulatory function of pancreatic β cell lncRNAs, we carried out loss-of-function experiments in a glucose-responsive human islet β cell line, EndoC-βH1 (Ravas-sard et al., 2011). We chose a human model because only some human lncRNAs are evolutionarily conserved (Demien et al., 2012; Morán et al., 2012; Okazaki et al., 2002; Pang et al., 2006), and we perturbed the function of lncRNAs through RNAi-based transcript knockdowns rather than genomic deletions because deletions could potentially disrupt cis-regulatory elements. We thus designed lentiviral vectors that contain RNA polymerase II-transcribed artificial microRNAs (hereafter referred to as amIRNA) with perfect homology to the target sequence to elicit target cleavage. The amIRNAs contain an artificial stem sequence targeting our lncRNA of choice as well as flanking and loop sequences from an endogenous miRNA to allow their processing as pre-miRNA by the RNAi pathway (Figure S1A). As a reference, we used the same strategy to knockdown TFs or lncRNA that had two valid amIRNA sequences, compared all ten possible combinations of two versus three control amIRNAs, and determined the number of differentially expressed genes (Figure 1A). As expected, knockdown of islet TFs consistently produced transcriptional phenotypes (Figure 1B). Remarkably, knockdown of 9 of the 12 islet lncRNAs also caused transcriptional changes (Figure 1B; Figure S1F). A more detailed analysis showed that some of the lncRNAs that presented knockdown phenotypes had visible effects on a neighboring gene, suggesting a possible cis-regulatory mechanism, although other such lncRNAs did not appear to affect neighboring genes and may thus function through trans-regulatory mechanisms (Figure 1E; Figure S1G).

These loss-of-function experiments with selected lncRNAs therefore suggested that lncRNAs can regulate the expression of pancreatic β cell genes.

Gene silencing using the RNAi pathway can theoretically lead to nonspecific gene deregulation. In our experimental model, a significant nonspecific result would occur when two unrelated amIRNAs elicited changes in a common set of genes that were not observed in the panel of control non-targeting amIRNAs. To assess the likelihood that two unrelated amIRNA sequences elicit such an effect, we studied the five sets of control (non-targeting) amIRNAs, compared all ten possible combinations of two versus three control amIRNAs, and determined the number of differentially expressed genes (Figure 1C). Likewise, for each TF or lncRNA that had two valid amIRNAs, we compared the two target-specific amIRNAs against all possible combinations of three control amIRNAs (Figure 1C). As seen in Figure 1D, control versus control comparisons generated a median of 16 (IQR = 15–22) differentially expressed genes, whereas all five TFs and six of the seven lncRNA knockdowns led to a significantly higher number of differentially expressed genes (Mann-Whitney test, p < 10^-4 for all lncRNA/TF versus control comparisons except HI-LNC75, p = 0.004, and HI-LNC76, p > 0.5). These results show that the observed phenotypes are unlikely to be caused by unspecific effects of amIRNAs and indicate that the sequence-specific inhibition of selected islet lncRNAs can result in transcriptional changes comparable in magnitude to the inhibition of well-established islet transcriptional regulators.

The primary function of β cells is to synthesize and secrete insulin in response to changes in glucose concentrations. Among the genes that showed functional dependence on lncRNAs, we identified numerous genes that are known to were near a protein-coding gene that has an important function in β cells. The lncRNAs had variable subcellular enrichment patterns (Figure S1B), and eight of the 12 lncRNAs had detectable transcripts in orthologous or syntenic mouse regions (Table S1; Morán et al., 2012). We then screened four amIRNA sequences for each of the 12 lncRNAs and identified two efficient (>50% knockdown) amIRNAs for seven lncRNAs and one efficient amIRNA sequence for the other five lncRNAs (Figure S1E). Two efficient amIRNAs were also obtained for five essential islet TFs (HNF1A, GLIS3, MAFB, NKX2.2, and PDX1). We thus transduced EndoC-βH1 cells with lentiviruses expressing each amIRNA. This was done in duplicate or in triplicate for lncRNAs that only had one efficient amIRNA. 80 hr post-transduction, RNA was harvested and hybridized to oligonucleotide microarrays (Figure 1A). For each target gene, we combined expression data from all knockdowns and compared them to the control transductions with five different control amIRNAs to identify genes that were differentially expressed at a significance level of p < 10^-3 (ANOVA) (Figure 1B).
regulate transcription or secretion in β cells, including RFX6, PDX1, CACNA1D, ATP2A3, ROBO1 and 2, PDE8A, ATP6AP1, KCNJ15, TRPM3, ERO1LB, and HADH (Figure 2A; Anderson et al., 2011; Li et al., 2010; Louagie et al., 2008; Okamoto et al., 2012; Smith et al., 2010; Tian et al., 2012; Varadi and Rutter, 2002; Wagner et al., 2008; Yang et al., 2013; Zito et al., 2010).

We therefore measured insulin content and glucose-stimulated insulin secretion (GSIS) in T antigen-excised EndoC-βH3 cells after knocking down four lncRNAs that showed the strongest transcriptional phenotypes (HI-LNC12, HI-LNC78, HI-LNC80, and HI-LNC71). Congruent with the broad transcriptional phenotype, we observed reduced insulin content and, consequently,
impaired glucose-stimulated insulin secretion for HI-LNC12, HI-LNC78, and HI-LNC71 knockdowns (Figure 2B). For HI-LNC78, a glucose-regulated islet transcript (Moran et al., 2012) that is orthologous to mouse Tunar and zebrafish megamind (linc-birc6) IncRNAs (Ullitsky et al., 2011), there was a reduction in GSIS after correcting for the reduction in insulin content (p = 0.002) (Figure S2A). To further validate these effects, the same IncRNAs were downregulated using antisense locked nucleic acid (LNA GapmeRs, Exiqon) GapmeRs, which also led to impaired insulin secretion after knockdown of HI-LNC12 and HI-LNC78 (Figure S2B). Taken together, IncRNA knockdown studies identified IncRNAs that modulate gene expression and, consequently, insulin secretion in a human β cell line.

Human Islet IncRNAs and TFs Regulate Common Gene Expression Programs

To gain insight into the expression programs that are regulated by islet-specific IncRNAs and TFs, we compared their knockdown gene expression phenotypes. We first assessed changes in gene expression occurring after knockdown of the different islet TFs and found high Pearson correlation values for all pairwise comparisons (r = 0.4–0.8, p < 10^{-27}) (Figure 3A; Figure S3). This finding is consistent with the notion that islet-specific TFs often bind to common genomic targets and function in a combinatorial manner (Pasquali et al., 2014; Qiu et al., 2002; Wilson et al., 2003). Interestingly, the transcriptional changes that occurred after the inhibition of several IncRNAs significantly correlate with those observed following inhibition of TFs (Figure 3A; Figure S3; see also a cluster analysis of TF- and IncRNA-dependent changes in Figure 3B). Some pairwise comparisons that illustrate this finding include HI-LNC78-dependent gene expression changes, which correlated highly with HNF1α- and MAFA-dependent changes (Pearson’s r = 0.87 and 0.89, respectively, p < 10^{-7}), and HI-LNC15-dependent changes, which correlated with those occurring after knockdown of NKX2-2 (r = 0.87, p = 10^{-32}) (Figure 3C). The results from these gene knockdown experiments therefore indicate that selected islet-specific IncRNAs and TFs can regulate common gene expression programs.

Islet TFs and IncRNAs Co-regulate Genes Associated with Enhancer Clusters

Recent studies have revealed that islet TFs regulate cell-specific transcription by targeting clusters of enhancers and, in particular, clusters with enhancers that are bound by multiple islet TFs (Pasquali et al., 2014). Enhancer clusters share many features with regulatory domains that have otherwise been defined as “stretch enhancers” or “superenhancers” (Pasquali et al., 2014; Pott and Lieb, 2015). Given that knockdown of islet IncRNAs and TFs suggested that they regulate similar genes, we asked whether islet IncRNAs also regulate enhancer cluster-associated genes. As expected, gene set enrichment analysis (GSEA) showed that genes with islet-enriched expression, genes associated with enhancer clusters, or genes associated with enhancers that are bound by multiple TFs were downregulated after knockdown of all five TFs, whereas this was not observed for ten control sets of genes expressed at similar levels (Figure 4; Figures S4A and S4B). Likewise, genes associated with enhancer clusters and those showing islet-specific expression were also enriched among genes that were downregulated after knockdown of HI-LNC12, 15, 30, 78, 80, 85, and 71 (Figure 4; Figures S4A and S4B). These results therefore indicate that islet-specific TFs and IncRNAs often co-regulate genes that are associated with enhancer clusters.

| A | β-cell function genes regulated by IncRNAs |
|---|---|
| HI-LNC12 | HI-LNC78 | HI-LNC71 |
| ADCY8 | KCNJ15 | ADCY8 | PGK1 | ERO1LB |
| ATP2A3 | Nfat5 | ATP2A3 | Nfat5 | HADH |
| ATP6A1 | PAX6 | ATP6A1 | PAX6 | KCNJ3 |
| CACNA1A | PCSK2 | CACNA1A | PCSK2 | TM4SF4 |
| CACNA1D | PDE8A | CACNA1D | PDE8A | PDX1 |
| CADM1 | ROBO1 | CADM1 | ROBO1 | VAMP3 |
| CADPS | ROBO2 | CADPS | ROBO2 | |
| CREBBP | SCIN | CREBBP | SCIN | |
| GNAS | TM4SF4 | GNAS | TM4SF4 | |
| HADH | TRPM3 | HADH | TRPM3 | |

Figure 2. Knockdown of IncRNAs Impairs Insulin Secretion

(A) Examples of genes known to play a role in β cell function regulated by islet IncRNAs.

(B) Glucose-stimulated insulin secretion was tested on T antigen-excised EndoC-βH3 cells after transduction with amiRNAs targeting the indicated IncRNAs or controls. Secreted or total insulin content was normalized to the number of cells per well and expressed as fold change over control amiRNA treatment at 2.8 mM glucose. Each bar represents an average from two independent amiRNA vectors and 12 separate wells from two independent experiments. Error bars represent SEM; **p < 10^{-3}, ***p < 0.01, *p < 0.05 (Student’s t test).
highly significant co-expression across human islet samples containing more than 100 genes, named M1–M25, that showed pancreatic islet samples. This identified 25 major gene modules (WGCNA) of RNA sequencing (RNA-seq) profiles from 64 human (Derry et al., 2010; Kim et al., 2001; Pandey et al., 2010; Segal et al., 2006) to reveal sets of genes that share functional relationships. Analogous approaches have been employed to reveal sets of genes that share functional relationships (Derry et al., 2010; Kim et al., 2001; Pandey et al., 2010; Segal et al., 2006). We next characterized the nature of these seven lncRNA-enriched co-expression modules. Five of these (M3, M7, M12, M13, M18, and M21) have been knocked down in EndoC-βH1 cells. Module M3, the largest of the seven lncRNA-enriched modules, featured gene ontology (GO) terms associated with prototypical islet cell functions and contained several islet TFs and lncRNAs (Figure 5E). In keeping with these findings, we found numerous instances of islet lncRNAs and known cell-specific TFs that showed a tight correlation of gene expression levels across human islet samples (Figures 5F; Figure S5C). These findings thus indicated that β cell-specific lncRNAs, TFs, and genes associated with islet enhancer clusters form part of common expression programs.

Further analysis is consistent with the notion that lncRNAs play a functional role in driving gene expression variation in the lncRNA-enriched co-expression modules. First, the subset of lncRNAs that were shown to regulate an adjacent gene in knockdown studies also exhibited a particular high co-regulation with the adjacent gene across islet samples (Figure 5A; Table S2). We next determined which co-expression modules contained islet lncRNAs. Rather than using our previously defined set of lncRNAs, this analysis was performed with a set of 2,373 β cell lncRNAs that were newly annotated using ~5 billion stranded RNA-seq reads pooled from 41 islet samples (Table S3; Figure S5A). β Cell lncRNAs were found to be enriched in seven pancreatic islet co-expression modules (M3, M7, M12, M13, M18, and M20) (Figure 5B).

We next characterized the nature of these seven lncRNA-enriched co-expression modules. Five of these (M3, M7, M12, M18, and M20) were enriched in genes associated with pancreatic islet enhancer clusters (Figures 5A–5C, marked in blue). Two other modules (M13 and M21) were enriched for ubiquitously expressed genes involved in mRNA translation and metabolic pathways (Figure S5B). Among the modules enriched in lncRNAs and enhancer clusters, three (M3, M7, and M18) were also enriched in islet-specific TF genes (Figure 5D), and two of these modules (M3 and M7) contained nine of the 12 lncRNAs that had been knocked down in EndoC-βH1 cells. Module M3, the largest of the seven lncRNA-enriched modules, featured gene ontology (GO) terms associated with prototypical islet cell functions and contained several islet TFs and lncRNAs (Figure 5E). In keeping with these findings, we found numerous instances of islet lncRNAs and known cell-specific TFs that showed a tight correlation of gene expression levels across human islet samples (Figures 5F; Figure S5C). These findings thus indicated that β cell-specific lncRNAs, TFs, and genes associated with islet enhancer clusters form part of common expression programs.
amiRNA-based perturbations in EndoC-βH1 cells and indicated that a group of islet IncRNAs and TFs form part of common transcriptional networks that target clusters of pancreatic islet enhancers (Figure 5G).

**Deregulation of \( \beta \) Cell IncRNAs in Human T2D**

The identification of functional IncRNAs led us to explore whether some IncRNAs are abnormally expressed in human T2D and might thus be relevant to the pathogenesis of this disease. We therefore analyzed our new set of 2,373 IncRNAs in a recently reported gene expression dataset that includes human islet samples from donors diagnosed with T2D or impaired glucose tolerance (IGT) (Fadista et al., 2014). Our results showed that despite the fact that gene expression across human islet donors is highly variable, the expression of 15 and 100 IncRNAs was significantly altered in islets from T2D and IGT versus non-diabetic donors respectively (adjusted \( p < 0.05 \)) (Figure S6 A; Figure 6 E). To test whether PLUTO could affect cis regulation of the PDX1 gene.

This observation suggested that PLUTO could affect cis regulation of the PDX1 gene.

To test whether PLUTO regulates PDX1, we first examined EndoC-βH1 cells after amiRNA-mediated knockdown of PLUTO RNA and found reduced PDX1 mRNA and protein levels (Figure 6 C). Similarly, knockdown of PLUTO RNA in dispersed primary human islet cells caused decreased PDX1 mRNA (Figure 6 D). To validate these experiments through a complementary approach, we used CRISPR interference (CRISPRi), which involves targeting guide RNAs (gRNAs) downstream of a gene’s transcriptional initiation site to block its transcription. Two independent gRNAs that targeted a region downstream of the PLUTO initiation site efficiently reduced PLUTO RNA levels relative to non-targeting gRNAs, and, in both cases, this led to decreased PDX1 mRNA expression (Figure 6 E). Therefore, perturbing either PLUTO RNA levels or its transcription leads to the same inhibitory effect on PDX1 mRNA.

The mouse Pdx1 locus also has an islet IncRNA (Pluto) that shows only limited sequence homology with human PLUTO. Pluto is also transcribed from the opposite strand of Pdx1 but is initiated from a promoter within the first intron of Pdx1 and, like PLUTO, spans a broad regulatory domain upstream of Pdx1 (Figure 6 C). Knockdown of Pluto RNA in the mouse β cell line MIN6 also led to decreased Pdx1 mRNA levels (Figure 6 E). These experiments therefore indicated that PLUTO regulates PDX1 mRNA in human β cell lines and primary islet cells, and an analogous effect was observed for the mouse IncRNA ortholog.

Consistent with this regulatory relationship, PLUTO and PDX1 RNA levels are highly correlated across islet samples (Pearson’s \( r = 0.86, p = 10^{-15} \); Figure 6 F), and knockdown of PDX1 and PLUTO in EndoC-βH1 cells resulted in the deregulation of a shared set of genes (Figures 6 G–6 J). Furthermore, PLUTO and PDX1 were found to be regulated with nearly identical dynamics in response to a shift in glucose concentration (4–11 mM) in mouse pancreatic islets (Figure 6 D). PLUTO and PDX1 therefore regulate a common program in pancreatic islets, and this is at least in part explained by the fact that PLUTO regulates PDX1.

**PLUTO Regulates PDX1 Transcription and Local 3D Chromatin Structure**

To assess the mechanisms underlying the function of PLUTO, we first examined whether PLUTO controls the stability or transcription of PDX1. Transcriptional inhibition experiments using Actinomycin D showed no significant differences in the stability...
significant changes in these characteristic active chromatin marks
of several enhancers within the cluster. Our results indicate no
acetylation as well as H3K4 mono- and tri-methylation levels at
PLUTO thus knocked down that it could regulate the chromatin state of active enhancers. We
(Figure 7 B).

Because PLUTO spans an enhancer cluster, we hypothesized
that it could regulate the chromatin state of active enhancers. We
thus knocked down PLUTO in β cells and measured H3K27 acety-
lation as well as H3K4 mono- and tri-methylation levels at several enhancers within the cluster. Our results indicate no sig-
nificant changes in these characteristic active chromatin marks
(Figure S7).

We next determined whether PLUTO affects the 3D contacts
between the enhancer cluster and the PDX1 promoter. Examination
of the PDX1 locus using quantitative chromatin conformation capture (3C) assays revealed that two far upstream enhancers (Figure 7C) showed reduced contacts with the
PDX1 promoter after PLUTO knockdown (Figure 7D). These
findings therefore show that PLUTO regulates the transcription of PDX1, a key pancreatic β cell transcriptional regulator, and that this is associated with its
ability to promote contacts between the PDX1 promoter and its enhancer cluster
(Figure 7E).

DISCUSSION

In the current study, we have tested the hypothesis that lncRNAs play a role in cell-specific gene regulation in pancreatic
β cells, a cell type that is central in the path-
ogenesis of human diabetes. We have
thus carried out, for the first time, a system-
atic analysis of the function of a set of
human β cell-specific lncRNAs. Our experiments revealed
several examples of β cell lncRNAs in which sequence-specific perturbation causes transcriptional and functional phenotypes. We have further shown that β cell-specific lncRNAs and TFs
regulate a common transcriptional network. Finally, we have
demonstrated that β cell-specific lncRNAs directly or indirectly
participate in the regulation of human enhancer clusters, which
are the major functional targets of islet-specific transcription
factors and key cis-regulatory determinants of islet cell trans-
scriptional programs (Pasquali et al., 2014). Importantly, these
conclusions are supported by concordant results from co-
expression network analysis and loss of function experiments.
These studies should be interpreted in light of previous evi-
dence indicating that a significant fraction of lncRNAs show
lineage-specific expression (Cabili et al., 2011; Derrien et al.,
2012; Goff et al., 2015; Guttman et al., 2011; Iyer et al., 2015;
Morán et al., 2012; Pauli et al., 2012). Our study extends
Our findings invite the question of what molecular mechanisms underlie the regulatory effects of b cell lncRNAs. LncRNAs have been proposed to control gene expression through diverse molecular mechanisms, including the formation of protein-specific interactions and scaffolds, RNA-DNA or RNA-RNA hybrids, the titration of miRNAs, and the modulation of 3D chromosomal structures (Rinn and Chang, 2012; Wang and Chang, 2011), whereas some transcripts currently defined as lncRNAs can theoretically encode for atypical small peptide sequences (Andrews and Rothnagel, 2014). Our knockdown and co-expression analyses have identified a subset of functional lncRNAs that appear to regulate a nearby gene, suggesting a lncRNA-based cis-regulatory mechanism, whereas others are likely to exert trans-regulatory effects. We focused on one

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**Figure 6. PLUTO Knockdown Decreases PDX1 mRNA**

(A) Downregulation of PLUTO (HI-LNC71) and PDX1 in islets from donors with T2D or IGT. Differential expression analysis was performed on control (n = 50) versus T2D (n = 10) or IGT (n = 15) samples. Boxplots represent expression normalized to the mean of control samples. Adjusted p values are shown.

(B) Schematic of the human PDX1 locus and its associated enhancer cluster. A 4C-seq analysis was designed to identify regions interacting with the PDX1 promoter region in EndoC-iiH1 cells. Red and orange vertical lines depict active and poised islet enhancers, respectively. F and R represent forward and reverse RNA-seq strands, respectively, and scales represent RPM. PLUTO (HI-LNC71) was generated from a de novo assembly of islet RNA-seq and differs from a transcript annotated in UCSC and RefSeq that originates from a PDX1 intronic region.

(C) Downregulation of PLUTO or PDX1 using amiRNAs resulted in reduced PDX1 mRNA and protein levels. EndoC-iiH1 cells were transduced with control (black), PLUTO (white), or PDX1 (turquoise) amiRNA vectors 80 hr prior to harvest. RNA levels were assessed by qPCR, normalized to TBP, and expressed as fold over control amiRNA samples (n = 4). For protein quantification, PDX1 levels were first normalized to the average of TBP and H3 levels and then compared with the control amiRNA sample.

(D) Downregulation of PLUTO in human islet cells results in reduced PDX1 mRNA levels. Islet cells were dispersed and transduced with amiRNA vectors (n = 3) as in (B).

(E) Downregulation of PLUTO in EndoC-iiH3 cells using CRISPRi also decreases PDX1 mRNA. EndoC-iiH3 cells were nucleofected with CRISPRi vectors 80 hr prior to harvest. RNA levels were assessed by qPCR and normalized to TBP and then to a control CRISPRi sample (n = 3).

(F) PLDX1 and PLUTO RNA levels were highly correlated in 64 human islet samples.

(G) Knockdown of PDX1 and PLUTO resulted in differential expression of genes with similar biological process annotations.

(H) GSEA showed that genes that were downregulated upon knockdown of PDX1 and PLUTO were enriched in genes whose enhancers were bound by PDX1 (red) in islets but not in ten control gene sets (black) that were expressed at similar levels as PDX1-bound genes.

(I) Knockdown of PDX1 and PLUTO resulted in differential expression of genes with similar biological process annotations.

(J) Examples of known PDX1-regulated genes that are also co-regulated by PLUTO in parallel knockdown experiments. mRNA levels were assessed as in (B). Error bars denote SEM; ***p < 10^-3, **p < 0.01, *p < 0.05 (Student’s t test).

previous findings by demonstrating a functional role of lncRNAs in lineage-specific TF networks.
Figure 7. PLUTO Regulates PDX1 Transcription and 3D Chromatin Structure

(A) The mRNA stability of PDX1 was unaffected by PLUTO knockdown. PDX1 mRNA was measured in control and PLUTO amiRNA knockdown in EndoC-H1 cells after Actinomycin D (ActD) treatment (n = 3). mRNA levels were presented as a percentage of levels observed at time = 0.

(B) Knockdown of PLUTO was carried as out in Figure 6B, and this led to reduced PDX1 transcription, as assessed by qPCR analysis of intronic PDX1 RNA levels using hydrolysis probes. Values were normalized to TBP mRNA and expressed as fold over the control amiRNA sample (n = 4).

(C) Schematic of selected epigenomic features of the PDX1 locus.

(D) PLUTO is required for 3D contacts between the PDX1 promoter and distal enhancers. SC analysis revealed that knockdown of PLUTO resulted in reduced contacts between the PDX1 promoter (anchor) and two enhancers (E1 and E2). Interaction signals were normalized to a control region on the PDX1 intron. CTL represents a negative control region that does not harbor interactions with the PDX1 promoter. Error bars denote ± SEM, and p values are from a Student’s t test.

(E) PLUTO knockdown resulted in impaired 3D contacts between the PDX1 promoter and its adjacent enhancer cluster, causing reduced PDX1 transcriptional activity.

(Bell and Polonsky, 2001; Flanagan et al., 2014; Zhou et al., 2008). The findings reported here therefore strengthen earlier suggestions that defects in β cell IncRNAs might contribute to the pathogenesis of human diabetes (Fadista et al., 2014; Morán et al., 2012) and warrant an assessment of whether they can be harnessed to promote β cell differentiation, function, or cellular mass.

EXPERIMENTAL PROCEDURES

Pancreatic Islets

Human islets used for RNA-seq and chromatin immunoprecipitation sequencing (ChIP-seq) were cultured with CMRL 1066 medium containing 10% fetal calf serum (FCS) before shipment, after which they were cultured for 3 days with RPMI 1640 medium containing 11 mM glucose and supplemented with 10% FCS.

Glucose-Stimulated Insulin Release

Glucose-stimulated insulin release was assayed in EndoC-H1 or EndoC-H3 cells as described previously (Benazra et al., 2015; Ravassard et al., 2011).

RNA Analysis

RNA was isolated with Tripure (Roche) and treated with DNase I (Sigma). qPCR was performed with SYBR green or Taqman probe detection (van Arensberg et al., 2010). See Table S4 for oligonucleotide and probe sequences.

amiRNA and CRISPRi Experiments

Lentiviral vectors carrying amiRNAs targeting TFs, IncRNAs, and non-targeting control sequences were transduced into the EndoC-H1 human β cell line as described previously (Castaing et al., 2005; Ravassard et al., 2011; Scharffmann et al., 2014).
Figure S1A illustrates the vector design. Oligonucleotide sequences are shown in Table S4. Non-transduced cells were assayed in parallel. Cells were harvested 80 hr post transduction for RNA extraction. For transduction of human islets, islets were first dispersed using trypsin-EDTA and gentle agitation. CRISPRi experiments were performed with two gRNAs designed to target PLUTO exon 1 or two unrelated intergenic control regions and transfected in EndoC-JH3 cells (Table S4).

Gene Expression Array Analysis
RNA was hybridized onto HTA2.0 Affymetrix arrays. RNA normalization was carried out using Expression Console (Affymetrix). Gene-based differential expression analysis was done using Transcriptome Analysis Console (TAC, Affymetrix). Enhancer cluster genes were defined by genes that were associated with clustered islet enhancers that show top 50 percentile binding by TFs (PDX1, FOXA2, NKX2-2, NKX6.1, and MAFB) as defined previously (Pasquali et al., 2014). Pancreatic islet gene sets used for enrichment analysis are shown in Table S5. A list of islet-enriched genes was generated as those with more than two SDs higher expression in human islets than the average expression in 16 human tissues (Table S5).

Differential Expression in IGT and T2D Islets
RNA-seq data have been described previously (Fadista et al., 2014). The samples were aligned to the hg19 genome using STAR aligner version 2.3.0 as described in the Supplemental Experimental Procedures. Quantification was carried out with HTSeq-Count 0.6.1, and differential expression analysis of lncRNA genes was done using DESeq2 1.10 (Table S3) using an adjusted p value threshold of 0.05.

3C
3C and 4C-seq was carried out as described previously (Pasquali et al., 2014; Tena et al., 2011) For real-time PCR quantification, readings were normalized to a control region within the PDX1 intron. Normalized values are expressed as a fraction of non-targeting amiRNA control sample. See Table S4 for oligonucleotide sequences.

Annotation of Islet lncRNAs
LncRNAs were annotated through de novo assembly of /C24 nucleotide sequences. A fraction of non-targeting amiRNA control sample. See Table S4 for oligonucleotide sequences.

Network Analysis
The WGCNA(v2) tool was used to build a co-trancriptional network based on mRNAs from 64 human islet RNA-seq samples.

ACCESSION NUMBERS
The accession number for the microarray data reported in this paper is GEO: GSE83619.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.11.016.

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