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Epigenetic regulation of the MEG3-DLK1 microRNA cluster in human Type 2 diabetic islets

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SUMMARY

Type 2 diabetes mellitus (T2DM) is a complex disease characterized by the inability of the insulin-producing β-cells in the endocrine pancreas to overcome insulin resistance in peripheral tissues. To determine if microRNAs are involved in the pathogenesis of human T2DM, we sequenced the small RNAs of human islets from diabetic and non-diabetic organ donors. We identified a cluster of miRNAs in an imprinted locus on human chromosome 14q32 that is highly and specifically expressed in human β-cells and dramatically down-regulated in islets from T2DM organ donors. The down-regulation of this locus strongly correlates with hyper-methylation of its promoter. Using HITS-CLIP for the essential RISC-component Argonaute, we identified disease-relevant targets of the chromosome 14q32 microRNAs, such as IAPP and TP53INP1 that cause increased β-cell apoptosis upon over-expression in human islets. Our results support a role for microRNAs and their epigenetic control by DNA methylation in the pathogenesis of T2DM.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a complex, multi-factorial disease, characterized by an insufficient pancreatic β-cell response to insulin resistance in peripheral tissues. As of September 2012, the World Health Organization estimated that at least 312 million people worldwide have T2DM. Several studies have indicated that T2DM has a high rate of familial aggregation (Drong et al., 2012; Meigs et al., 2000; Nolan et al., 2011). However, genetic risk loci identified by standard genetic and genome wide association approaches account for less than 10% of the observed heritability. These results have led to speculation that epigenetic effects may also play a role in the development of T2DM. Indeed, there is suggestive evidence that diet and intrauterine environment, among other factors, may induce chromatin changes that lead to aberrant gene expression and subsequent disease (Bramswig et al., 2013; Drong et al., 2012).

MicroRNAs (miRNAs), short non-coding RNAs that post-transcriptionally regulate gene expression, have emerged as a strong molecular candidate in several complex diseases, in part due to their ability to simultaneously regulate the expression of hundreds of target mRNAs (Mendell and Olson, 2012). While several recent studies have suggested a role for miRNAs in human pancreatic islet and β-cell function (Klein et al., 2013; van de Bunt et al., 2013), none have profiled the miRNA transcriptome of islets obtained from diabetic donors. To address this knowledge gap, we performed high-throughput sequencing of small RNAs and have identified several miRNAs as significantly differentially expressed between islets isolated from non-diabetic and T2DM organ donors. Strikingly, included among the miRNAs down-regulated in T2DM donors' islets was a cluster of maternally expressed miRNAs mapping to an imprinted locus on human chromosome 14q32. Our results demonstrate that the DLK1-MEG3 miRNA cluster is highly and specifically expressed in human β-cells, but strongly repressed in islets from T2DM donors. Furthermore, we identify an epigenetic modification at this locus that correlates with its expression in human diabetic donors' islets. Using high-throughput sequencing of cross-linked and immunoprecipitated RNA (HITS-CLIP) we have identified targets of Chr 14q32 miRNAs, such as IAPP and TP53INP1, with known association to the pathogenesis of T2DM. Additionally, we identified a subset of sequences within CLIP libraries that are generated by the ligation of miRNAs to their targets while in complex with Argonaute. These reads, called chimeric reads, allow for the direct identification of miRNA:target relationships *in vivo*.

RESULTS

Differentially expressed miRNAs in T2DM human islets

To determine the miRNA transcriptome of the mature human islet, we isolated the small RNA fraction from islets of three non-diabetic and four T2DM organ donors (Donor information available in Table 1). We employed ultra-high throughput sequencing and obtained more than 100 million sequence reads, allowing us to identify over 800 miRNAs expressed in the human endocrine pancreas (Supplementary Table S2). In order to verify that our sequence reads represented miRNAs and not degraded mRNAs, we aligned them to the RefSeq database (Pruitt et al., 2012). As shown in Supplementary Fig S1a, less than 20% of the reads in the miRNA size range aligned to mRNAs, while more than 85% matched precursor miRNAs, confirming that our small RNA preparation was indeed highly enriched for true miRNAs. To assess the abundance of each mature miRNA, we aligned all sequence reads to known miRNA precursors obtained from miRBase (Kozomara and Griffiths-Jones, 2011). The 15 most highly expressed miRNAs in human islets from non-diabetic and T2DM donors are shown in Fig 1a and b. Hsa-miR-375, which was shown to be highly expressed in developing and mature human islets (Bolmeson et al., 2011; Joglekar et al., 2009; Landgraf et al., 2007), is the fourth–most abundant miRNA in human islets with over 100,000 reads

per million (RPM). Similarly, miRNAs miR-103 and -107, that are important regulators of insulin sensitivity in the livers of obese mouse models were also identified as highly abundant in human islets (Trajkovski et al., 2011). The abundance of individual miRNAs varied greatly, from 1 to 229,000 RPM; however, 123 of these miRNAs were expressed at more than 100 RPM (Supplementary Table S2).

Comparative analysis of samples from T2DM donors and non-diabetic donors identified 15 miRNAs with significantly differential expression (Fig 1c). The expression levels of these miRNAs were highly consistent between samples and were sufficient to clearly cluster the samples as T2DM and non-T2DM, as shown in Fig 1d. The differential expression was validated for 9 of these miRNAs by Taqman qRT-PCR on islets from 16 T2DM and 18 non-diabetic donors (Supplementary Fig S1b and c). MiRNAs miR-204 and -184, which have previously been reported to be β-cell enriched (Klein et al., 2013; van de Bunt et al., 2013) are not differentially expressed between human islets from T2DM and non-T2DM organ donors (Supplementary Table S2), ruling out the possibility that the observed differential expressions are driven by changed islet composition between the two groups. Expression of these miRNAs did not show a significant correlation with age, sex or body mass index (BMI) (Supplementary Fig S1e, f and g).

Of the miRNAs that were identified as differentially expressed between T2DM and non-diabetic donor islets, several have been previously implicated in diabetes and β-cell function. For example, miR-7 is a well-characterized islet microRNA that is expressed in the endocrine cells of the developing and adult human pancreas (Correa-Medina et al., 2009). MiR-7 has been shown to regulate insulin expression during the early stages of mouse pancreatic embryogenesis (Nieto et al., 2012) and also negatively affects β-cell proliferation in murine and human islets (Wang et al., 2013). Thus, we have identified several new and previously described microRNAs as differentially expressed between T2DM and non-T2DM donors' islets.

Decreased expression of the imprinted MEG3 microRNA cluster in T2DM islets

Strikingly, of the ten miRNAs that were significantly down-regulated in T2DM islets, seven are derived from the imprinted *MEG3/GTL2* locus at human chromosome 14q32. Genomic imprinting refers to the biased expression of genes from either the paternally or maternally inherited chromosome, rather than the more common biallelic expression. Apart from the aforementioned miRNAs, this imprinted locus contains maternally expressed snoRNAs, the non-coding RNA genes *MEG3*, *MEG8* and antisense *RTL1*, as well as the paternally expressed genes *DLK1*, *RTL1*, and *DIO3* (Cavaille et al., 2002; Charlier et al., 2001; Wylie et al., 2000). Decreased expression of *MEG3* and the nearby miRNAs has been associated with numerous diseases, notably hepatocellular carcinoma, acute myeloid leukemia, and ovarian cancer (Benetatos et al., 2012), but not T2DM.

Since the maternally expressed non-coding RNAs in this locus are likely all processed from the same primary transcript (Seitz et al., 2004) (see additional evidence in Fig 2c below), we asked whether other miRNAs in this cluster were also expressed at lower levels in T2DM donors' islets. As shown in Supplementary Fig S1d, this was indeed the case, and these results were confirmed by Taqman qRT-PCR in a larger cohort of islet samples (Fig 1e). *MEG3* was also found to be down-regulated in islets from T2DM donors compared to non-diabetics (data not shown). Thus, we have identified an imprinted, maternally expressed cluster of non-coding RNAs to be down-regulated in islets obtained from T2DM donors.

The MEG3-DLK1 cluster of miRNAs is specifically expressed in human β-cells

To characterize the expression profile of the DLK1-MEG3 cluster of miRNAs in the major human islet cell populations, we applied FACS to sort highly purified human α- and β-cells (Dorrell et al., 2008) and performed high-throughput sequencing of small RNAs (Supplementary Fig S2 and Supplementary Table S3). The expression levels of the miRNAs in the 14q32 cluster were, on average, 16-fold higher in β-cells when compared to α-cells (Fig 2a and b). This is consistent with previous reports that utilized different sorting strategies and identified some members of the Chr 14q32 cluster of miRNAs as enriched in human β-cells compared to α-cells (Klein et al., 2013) and whole islets (van de Bunt et al., 2013). Expression of the long non-coding *MEG3* RNA was also found to be 20-fold higher in β-cells compared to α-cells (Dorrell et al., 2011), further supporting our results.

To understand the epigenetic landscape that may explain the cell-type specific expression of this locus in human α- and β-cells, we performed ChIP-Seq for several histone modification marks in enriched human α- and β-cell populations (Bramswig et al., 2013). While the MEG3 promoter was bivalently marked by the activating histone H3 lysine 4 trimethylation (H3K4me3) and the repressive lysine 27 trimethylation (H3K27me3) modifications in glucagon secreting α-cells, insulin secreting β-cells showed a dramatic decrease in H3K27me3 and were only marked by H3K4me3 at this region (Fig 2c). Thus, the observed histone modification marks at the promoter of MEG3 and its associated miRNAs strongly correlate with its cell-type specific expression. Taken together, the 14q32 locus is highly and specifically expressed in β-cells.

Epigenetic regulation of the MEG3 promoter in T2DM islets

To understand the molecular mechanism of the down-regulation of the maternal RNAs in the DLK1-MEG3 locus, we considered the possibility that this may be a consequence of the high glucose conditions that the cells are exposed to in the islets of T2DM organ donors. To test this notion, we cultured islets from non-diabetic donors in different glucose conditions for a prolonged period of time and measured the changes in expression of the miRNAs located in this cluster by high throughput sequencing of small RNAs from each group. No significant change was detected in the expression of these miRNAs (Supplementary Fig S3a), suggesting that the expression of this cluster is not regulated acutely by glucose.

These findings prompted us to consider other genetic and epigenetic explanations for the observed decrease in miRNA levels at the Chr 14q32 locus in T2DM donors' islets. The imprinted status of the maternally expressed RNAs of the DLK1-MEG3 locus is determined by the methylation of two differentially methylated regions (DMRs), the first located 13 kb upstream (termed "IG-DMR") and the second 1.5 kb upstream of the transcription initiation site of MEG3, overlapping with the MEG3 promoter (termed "MEG3-DMR") (Fig 3a) (Kagami et al., 2010; Murphy et al., 2003). Hypermethylation of either of these DMRs has been concomitantly observed with decreased expression of the maternal transcript (Kagami et al., 2010). Using methylation-specific PCR primers designed for the MEG3-DMR (Murphy et al., 2003), we tested for differences in DNA methylation between T2DM and non-T2DM donors' islets. While islets from non-T2DM donors showed the predicted equal abundance of the methylated and unmethylated PCR products, we observed a decreased intensity of the unmethylated product in islets from donors with T2DM (Fig 3b, Supplementary Fig S3b).

To assess this difference at base resolution, we designed quantitative sequence-specific pyrosequencing assays to measure CpG methylation of both DMRs. No difference in methylation levels was detectable at the IG-DMR (Fig 3c, average tested CpG methylation decreased by 4.6% in T2DM islets, p-value = 0.35). In contrast, we observed significantly

increased methylation levels at the MEG3-DMR (Fig 3d, average increase of 14.5% across the tested CpGs, p-value < 0.01), consistent with the decreased expression of the maternally expressed genes under its control. We detected no difference in the methylation levels of α-cells sorted from T2DM and non-T2DM donors, suggesting that the observed hyper-methylation in T2DM islets is unlikely to arise from this cell population (Supplementary Fig S3c). These results provide a compelling example of an epigenetic modification that is associated with altered gene expression in islets from T2DM donors.

Targets of Chr 14q32 miRNAs are critical to β-cell health and function

In order to assess the contribution of specific differentially expressed islet miRNAs to T2DM, an understanding of the mRNAs they target is necessary. Therefore, we performed HITS-CLIP for Argonaute (Chi et al., 2009; McKenna et al., 2010), which forms part of the RNA-induced silencing complex (RISC) that mediates miRNA action. By cross-linking the protein components of the RISC to the paired miRNA and mRNA simultaneously, and isolating these RNA species by immunoprecipitation of Argonaute, we identified miRNA-targeted mRNAs in human islets using high-throughput sequencing (Fig 4a). From these deep-sequencing libraries, we identified 12,492 mRNA footprints, and 456 mature human miRNAs as Argonaute-associated in human islets. The mRNA footprints were highly enriched (96.85%) for seed sequences of the corresponding miRNAs identified by HITS-CLIP. Although most models of miRNA function propose seed sequence binding preferentially at the 3'UTR of the target mRNA (Friedman et al., 2009), global analysis of our HITS-CLIP data demonstrated that miRNAs bind their targets in human islets throughout the transcript, with comparable levels at the coding sequence (CDS) (Students t-test, p-value = 7.00E-16) and 3'UTR (Students t-test p-value = 5.26E-17), similar to what has previously been described for other tissues (Chi et al., 2009; Forman et al., 2008; McKenna et al., 2010) (Fig 4b,c). Among the mRNAs targeted by the RISC complex in human islets were several encoded by genes known to be essential for islet function.

Of the 54 miRNAs encoded by the 14q32 locus, 38 were detected in our miRNA library of HITS-CLIP in human islets. These 38 miRNAs are predicted to target mRNAs transcribed from 1,784 genes that were detected in the target library (Supplementary Table S4). Since the 14q32 locus is primarily expressed in β-cells, we further filtered these 1,784 potential target mRNAs to those expressed preferentially in human β-cells by intersecting our HITS-CLIP dataset with β-cell RNA-seq expression data (Bramswig et al., 2013). The filtered list contained 717 target mRNAs for the 38 miRNAs detected in our HITS-CLIP library, and 996 targets for all 54 mature miRNAs expressed from this locus (Supplementary Table S5).

Since the expression of the MEG3 locus is down-regulated in islets of T2DM donors, we were particularly interested in targets with known detrimental effects to islet function when expressed at higher levels than normal. Several mRNAs identified by our analysis are relevant to diabetes pathogenesis, such as islet amyloid polypeptide (IAPP), the major component of the amyloid deposits in pancreatic islets that cause increased β-cell apoptosis in T2DM (Butler et al., 2003; Hoppener and Lips, 2006; Hull et al., 2004). In order to test if the 14q32 locus miRNAs indeed target the 3'UTR of the IAPP mRNA directly, we performed co-transfection assays of 3'UTR luciferase reporter constructs with expression plasmids for the relevant microRNAs. The expression of the IAPP 3'UTR luciferase construct was suppressed by 20% upon co-expression of miR-376a and miR-432, but not empty vector, confirming the direct targeting relationship (Fig 4d). This finding suggests that the repression of the 14q32 locus miRNAs in the β-cells of T2DM donors results in the mis-regulation of key biological processes that contribute to the dysfunction of β-cells in T2DM.

Discovery of chimeric reads

While performing the alignment of the HITS-CLIP target library to its reference sequences, we discovered a unique class of sequences, termed chimeric reads, that partially mapped to both miRNAs and target mRNAs simultaneously (Supplementary Fig S4). Chimeric reads most likely arise from an occasional ligation event of miRNA and mRNA molecules while they are both associated with Argonaute in the RISC complex (Fig 4a). Though few in number (0.27% of all trimmed reads), these reads are an invaluable source for miRNA and target pair information, as the ligation event will only occur between molecules in close proximity to each other. This was confirmed by the fact that the miRanda target prediction algorithm identified base pairing between miRNAs and the fused mRNAs significantly more often than would be expected by chance ($p < 1E-300$). The fifteen most abundant mRNAs and miRNAs found in such chimeras are listed in Fig 5a and c, respectively. Many relevant and highly expressed human islet transcripts were found in chimeric reads, such as glucagon, INS-IGF2, chromogranin A and B, among others. We also determined the mRNAs that are highly enriched in chimeric reads, relative to their overall abundance, as these mRNAs are more likely to be highly regulated by miRNAs (Schug et al., 2013). The fifteen most highly enriched mRNAs in chimeric reads, relative to abundance are shown in Fig 5b. A list of chimeric reads found by HITS-CLIP in human islets can be found in Supplementary Table S6.

Gene ontology (GO) analysis of all human islet miRNA targets identified by both HITS-CLIP (footprint of >150 RPKM), and the chimeric reads analysis (>50 reads), revealed a significant enrichment of biological processes such as “protein localization and transport”, “protein ubiquitination”, and “regulation of cell death” (Fig 5d), suggesting that mRNAs involved in these processes in human islets are highly regulated by miRNAs. MiRNAs were found to form chimeras predominantly with the 3’UTR regions of the target mRNA compared to the 5’ UTR and coding region (Fig 5e and f).

Using this information, we identified several additional targets of miRNAs in the 14q32 locus, including the ‘p53-induced nuclear protein 1’, or TP53INP1 (Fig 6a). *TP53INP1* is the nearest gene to a T2DM risk-associated single nucleotide polymorphism in individuals of Caucasian (Voight et al., 2010) and North African Arab descent (Cauchi et al., 2012). TP53INP1 plays a crucial role in p53 dependent apoptosis (Okamura et al., 2001), and an increase in its expression in pancreatic β-cells is associated with increased cell death (Zhou et al., 2012). As expected, we observed an increase in TP53INP1 mRNA levels in T2DM donors’ islets compared to non-diabetic donors by both microarray (data not shown) and qPCR, although these data did not reach statistical significance (Fig 6b). The variability in target mRNA expression is a reflection of the heterogeneity of our donor samples. To address this issue further, we plotted miR-495 and TP53INP1 mRNA levels for each T2DM islet sample and observed a strong inverse correlation between the two (R^2=0.74, Fig 6c). Next, we validated the miR-495 and TP53INP1 targeting relationship using luciferase reporter assays as these assays provide a readout of miRNA effects on the mRNA and protein level (Fig 6d). We observed a 20% decrease in luciferase activity in the presence of miR-495 mimic, but not scramble mimic. To further test this targeting relationship *in vivo*, we constructed tough decoy RNAs (Haraguchi et al., 2009) for miR-495 (TuD495) in a lentiviral backbone to suppress miR-495 activity in human islets. We observed a 1.3 fold increase in TP53INP1 mRNA levels upon TuD495 transduction relative to control vector, TuDctrl (Fig 6e, p= 0.007), similar to the increase observed in Fig 6b. Onecut1, a previously published target of miR-495 (Simion et al., 2010) also increased to a similar extent (data not significant). In summary, de-repression of TP53INP1 as a consequence of increased miR-495 levels in β-cells from T2DM donors is likely to contribute to their increased

susceptibility to apoptotic stimuli. These results further underscore the value of the chimeric sequences in identifying miRNA targets.

DISCUSSION

MiRNAs have been shown to play a central role in the development and progression of several diseases (Mendell and Olson, 2012). To identify the miRNAs that are key to the pathogenesis of T2DM, we sequenced the small RNAs of islets obtained from healthy and T2DM organ donors. Of the miRNAs that were differentially expressed in T2DM islets, we identified the maternally expressed, imprinted cluster of non-coding RNAs on human chromosome 14q32 as down-regulated. Our data on the H3K4me3 and H3K27me3 histone modification marks, combined with the miRNA expression data on sorted α- and β- cells (supported by previous expression studies (Bolmeson et al., 2011; Klein et al., 2013; van de Bunt et al., 2013)) indicate that this complex maternally-expressed gene is primarily transcribed in the insulin secreting β-cells, compared to other pancreatic islet cell types.

Repression of this miRNA cluster is strongly correlated with hyper-methylation of the MEG3-differentially methylated region in T2DM islets, demonstrating an epigenetic alteration associated with T2DM. A report from Ling *et al* has shown that a 6% increase in DNA methylation at the *PPARGC1A* promoter was negatively correlated with insulin gene expression and secretion (Ling et al., 2008), reaffirming the detrimental functional consequences of aberrant methylation in T2DM islets. Although our results suggest that the change in expression of the miRNAs is unlikely to be induced by high glucose conditions, we cannot rule out the possibility that the observed hypermethylation at the MEG3 promoter may be a secondary effect of the diabetic state. Our evidence of loss of imprinting at the differentially methylated region of this locus in T2DM donor islets suggests that modifications at this region markedly increase susceptibility to disease, since imprinted loci are functionally haploid. These results necessitate the study of other imprinted loci, particularly those that are strongly associated with risk for T2DM, such as the maternally expressed genes *KLF14* and *KCNQ1* (Kong et al., 2009; Travers et al., 2012; Voight et al., 2010).

We have integrated high-throughput sequencing of the miRNA transcriptome of the human islet with HITS-CLIP of Argonaute-associated RNAs. Within the CLIP libraries, we identified a unique fraction of sequences, termed chimeric reads, that represent miRNAs fused to their respective targets while in a complex with Argonaute *in vivo*. Chimeric reads are proposed to result from the ligation of RNA molecules that are stably base-paired (Kudla et al., 2011), such as miRNAs and their targets, and were shown to form strong secondary structures with lower mean folding energies than non-chimeric reads of the same length (Kudla et al., 2011). By combining these datasets, we have identified islet specific-miRNAs and their mRNA targets that are mis-expressed in T2DM. Several of these targets, such as IAPP and TP53INP1, have well-established associations with T2DM pathogenesis, and their upregulation is strongly linked to β-cell dysfunction and increased cell death. This suggests that upon repression of the Chr 14q32 miRNA cluster, several pro-apoptotic factors, whose expression is normally tightly regulated, become activated. De-repression of this normally silent genetic locus, together with other risk factors, can result in increased β-cell death and T2DM pathogenesis. In sum, our results provide strong evidence for a role of microRNAs and epigenetic modifications, such as DNA methylation, in the pathogenesis of T2DM.

EXPERIMENTAL PROCEDURES

Human Islets

Human islets and relevant donor information including age, gender, diabetes status, hemoglobin A1c and BMI were obtained from the Islet Cell Resource Center of the University of Pennsylvania, the NIDDK-supported Integrated Islet Distribution Program (iidp.coh.org) and the National Disease Research Interchange. The donor's diabetes status was defined by the patient's medical record, and, when available, the hemoglobin A1c. Donor information is listed in Supplementary Table S1. Small RNA was isolated from the islets by using the mirVana miRNA Isolation Kit (Ambion Cat# AM1560).

Primers

All primers used in this study are listed in Supplementary Table S7.

miRNA sequencing & comparison

The isolated microRNA from seven samples (three from donors without diabetes and four with type 2 diabetes) were prepared for sequencing using the Illumina protocol Preparing Samples for Analysis of Small RNA (Illumina FC-102-1009). Sequencing of the amplified libraries was performed on an Illumina Genome Analyzer II (Illumina FC-104-1003). Sequenced libraries were processed as described in the Supplementary Methods. We used a false discovery rate of 20% and a minimum fold change of 1.5× to identify differentially expressed miRNAs.

Similarly, sorted cells were obtained by FACS sorting dispersed human islets as described before (Bramswig et al., 2013) and RNA was isolated from the α- and β-cell enriched fractions using the miRVana miRNA Isolation kit (Ambion). 3μg of RNA was used for library preparation using TruSeq Small RNA sample preparation kit (Illumina) and Pippin Prep (Sage Science) for size selection using 3% Cassette (CSD3010). Small RNA libraries from sorted α- and β- cells were sequenced to 50bp on an Illumina hiSeq2000. The miRNAs identified as differentially expressed in this study are listed in Supplementary Table S2.

All miRNA sequencing datasets have been deposited into NCBI GEO under accession number GSE52314.

Taqman qRT-PCR

Real-time quantitative polymerase chain reaction (qRT-PCR) was performed as previously described(Zahm et al., 2012). Briefly, 10ng of total RNA was reverse-transcribed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Cat. No. 4366596) and RT primers from the respective TaqMan MicroRNA Assay kit (Applied Biosystems, probe numbers listed separately in supplementary Table S7). qRT-PCR was performed on a Agilent Mx3005P using the TaqMan Universal PCR Master Mix (Applied Biosystems part number 4304437) and the TaqMan probe from the respective TaqMan MicroRNA Assay kit. Tissue miRNA levels were normalized to endogenous snoRNAs RNU44 and RNU48.

DNA methylation analysis

Genomic DNA or chromatin was extracted from 5 non-diabetic donors and 9 T2DM donor's islets using All Prep DNA/RNA kit (QIAGEN). 325 nanograms of extracted DNA (or unsonicated chromatin input) were bisulfite treated with the EpiTect Bisulfite kit (QIAGEN) and eluted in 20μl of Buffer EB. PCR and sequencing primers were designed using the PyroMark assay design software version 2.0 (QIAGEN, sequences listed in Supplementary Table S7) to cover CpGs at the IG-DMR and MEG3-DMR. Bisulfite-converted DNA was

amplified by PCR using the PyroMark PCR kit (QIAGEN) at 95°C for 15 mins followed by 45 cycles at 95°C for 15s, 57°C for 30s and 72°C for 15s. Biotinylated PCR products were immobilized onto streptavidin-coated sepharose beads (GE Healthcare) and DNA strands were separated using PyroMark denaturation solution (QIAGEN), washed and then neutralized using a vacuum prep station (QIAGEN PyroMark Q96 workstation). After annealing the sequencing primer to the immobilized strand, pyrosequencing was performed on the PyroMark Q96 MD (QIAGEN) using PyroMark Gold CDT kit (QIAGEN) according to the manufacturer's instructions. Data were analyzed using the Pyro Q-CpG software program (QIAGEN). Methylation specific PCR was performed as previously described(Benetatos et al., 2010).

HITS-CLIP experiment and sequencing data processing

HITS-CLIP was performed as previously reported, using the monoclonal Argonaute antibody 2A8 (Chi et al., 2009; McKenna et al., 2010). Human Islet samples were coarsely homogenized with a Dounce homogenizer and cross-linked three times on ice at 400 mJ/cm2. Both the miRNA library and mRNA library were sequenced on a Hi-Seq 2000 following standard protocols to a length of 100 nucleotides to yield 120,901,521 and 47,026,559 reads, respectively. Reads from both libraries were preprocessed and mapped to the human genome (USSC hg19 assembly) using a previously described analysis pipeline (Li et al.; Zheng et al.). Potential miRNA-target pairs were predicted between the 456 mature human miRNAs (miRBase 18) detected in our miRNA library and 12,496 Ago footprints using the miRanda program (v3.3a) with non-default parameter as "–en −10". Additionally, we overlaid our mRNA targets of miRNAs encoded by the Chr 14q32 locus to β-cell specific expressed transcripts using published RNA-seq data (Bramswig et al., 2013); in this study, β-cell specific expressed transcripts were defined as those with substantial expression (RPKM≥1) and having higher expression than α-cells and exocrine cells. All HITS-CLIP library sequencing data have been deposited into NCBI GEO under accession number XYZ.

The detailed analysis method is described in the Supplementary Methods section.

Chimeric reads analysis

A schematic of the chimeric reads detection is shown in Supplementary Fig S4. Among 47,026,559 total raw reads with 100bps in length, we found 26,542,918 reads whose length is larger than 15bps after trimming the adapter and any reported bases past the adapter. We checked if a read is a hybrid by mapping the sequence using BLAT (Kent, 2002) to identify if a portion of sequence mapped to the genome. From the BLAT results, we selected the result with the maximum number of matched bases among the results with the minimum number of mismatches. We discarded the sequence if the unmapped portion of the sequence has a length smaller than 5, resulting in 3,861,560 reads. As BLAT cannot map sequences less than 20bps, we applied Bowtie (Langmead et al., 2009) to the remaining portion of the read and collected the reads uniquely mapped to the genome, resulting in 1,233,580 reads (2.6% of the total 47,026,559 reads).

Next, we asked if the identified hybrid reads show miRNA-mRNA sequence match. For this, we used the mature miRNA sequence of the identified miRNA and checked if mRNA sequence has its sequence match pair using miRanda (John et al., 2004). As the mRNA portion of the sequence is short and may not cover the matched sequence, we extended the range of the identified mRNA portion to 10bps. We found 58,970 sequence matches (out of 127,512 hybrid reads) when we used 10bp extension.

The Ago-associated regulatory load for mRNAs in chimeric reads was determined by the ratio of sequence read counts to overall mRNA abundance (human islet RNA-seq results obtained from (Moran et al., 2012)).

Dual luciferase reporter assay

6×10^4 HEK293FT cells were seeded into 24-well plates. After 24hrs, cells were transfected with either miRNA expression plasmid or mimic along with dual luciferase reporter plasmid. Each construct was transfected in four replicate wells and repeated in three independent experiments. Cells were lysed and processed using Promega Dual–Luciferase Reporter Assay system, as per manufacturer's recommendation. Firefly and renilla luciferase activities were measured on a Synergy HT (KC4 v3.4 software, Bio-Tek Instruments, Inc.) using Stop and Glo reagents (Promega), according to the manufacturer's instructions. Relative light units were calculated as the ratio of renilla to firefly luciferase activity, and the reporters were normalized between the control expression and the empty pMirglo values for a given treatment.

Lentiviral transduction of human islets

Tough decoys were synthesized as described in (Haraguchi et al., 2009) and subcloned into a pSlik-Venus lentiviral backbone. Lentivirus was prepared and titrated by the Wistar Protein Expression facility. Viral titers were in the range of 5×10^7–1×10^9 TU/ml. Groups of 200–250 islets were transduced overnight with 5×10^3 TU/islet with 4μg/ml of polybrene. Media was replaced every day and RNA was extracted 72 hours post transduction for RT-qPCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ARTICLE HIGHLIGHTS

- An imprinted MEG3-DLK1 miRNA cluster is down-regulated in human T2DM islets.
- The MEG3 promoter is hyper-methylated in islets from T2DM organ donors.
- >700 β-cell specific mRNA targets of these miRNAs were identified by HITS-CLIP.
- Targets are involved in β-cell apoptosis and contribute to T2DM pathogenesis.

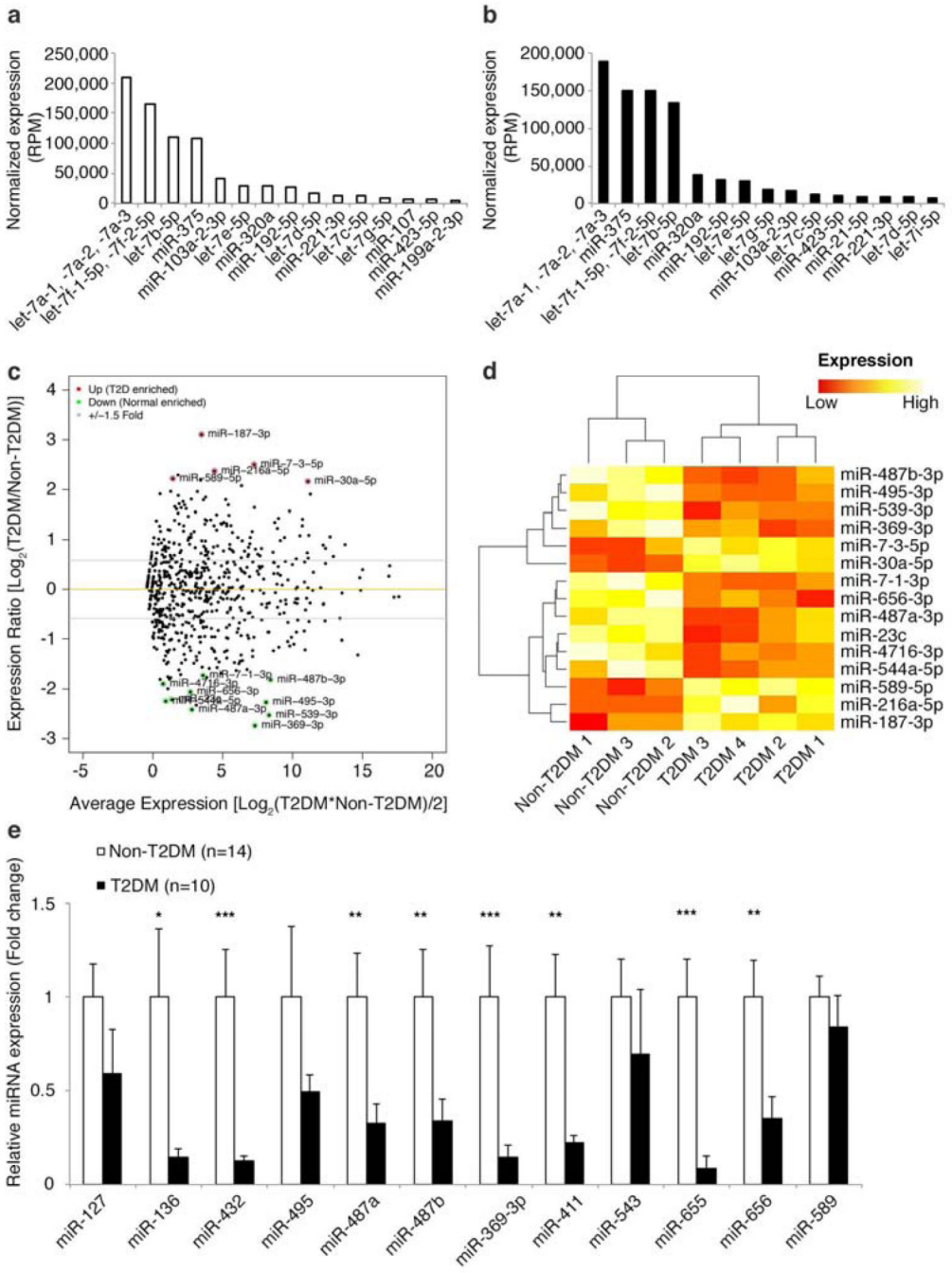

Fig 1. The imprinted chromosome 14q32 miRNA cluster is down-regulated in T2DM islets

Expression levels of the 15 most abundant miRNAs in (a) three non-diabetic and (b) four T2DM human islets as identified by small RNA sequencing. (c) Differentially expressed miRNAs between non-T2DM (n=3) and T2DM (n=4) islets, identified by a FDR of 20% and minimum fold change of 1.5×. MiRNAs up- and down-regulated in T2DM islets are highlighted, and miRNAs belonging to the Chr 14q32 cluster are underlined. (d) Distribution of expression levels of miRNAs identified as significantly differentially expressed by small RNA sequencing across clustered samples. (e) Relative expression of miRNAs in the Chr 14q32 cluster as determined by Taqman qPCR of 14 non-T2DM and 10

T2DM human islets. p-value calculated using two-tailed Student's t-test. *p<0.05, **p-value<0.01, ***p-value<0.005. +/− SEM. See also Figure S1 and Table S2.

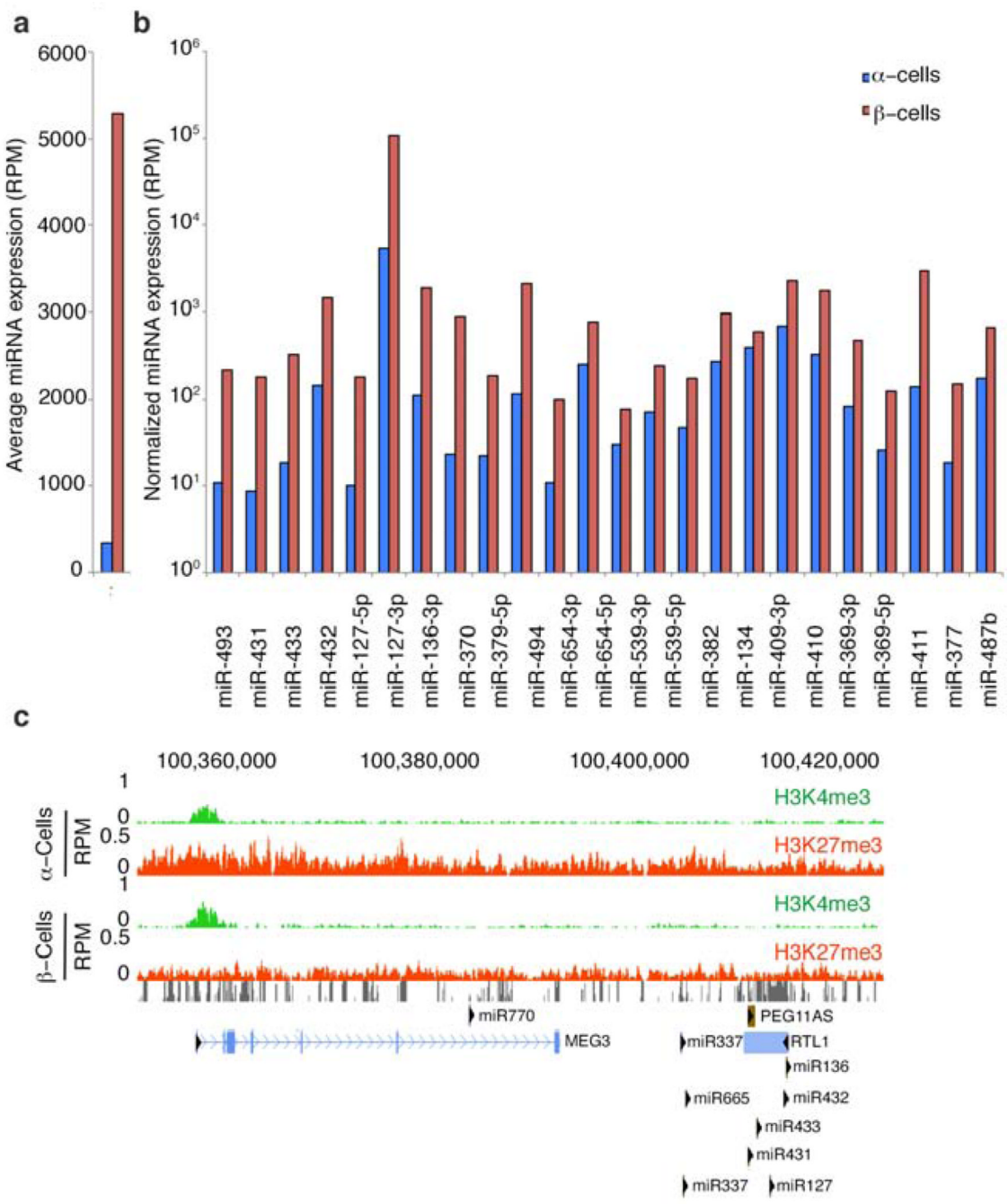

Fig 2. Chr 14q32 miRNAs are highly and specifically expressed in human β-cells

(a) Average expression of Chr 14q32 cluster miRNAs in human α- and β- cells. (b) Expression of Chr 14q32 miRNAs (minimal expression 50 reads per million (RPM)) in sorted human α- and β- cells. (c) Genome browser image of histone modification marks H3K4me3 (n=4) and H3K27me3 (n=3) at the MEG3 promoter (chromosomal location marked on top) of sorted α- and β- cells from healthy human donors. Grey bars represent sequence conservation. Positions of the miRNA cluster and other nearby transcripts are shown. See also Figure S2 and Table S3.

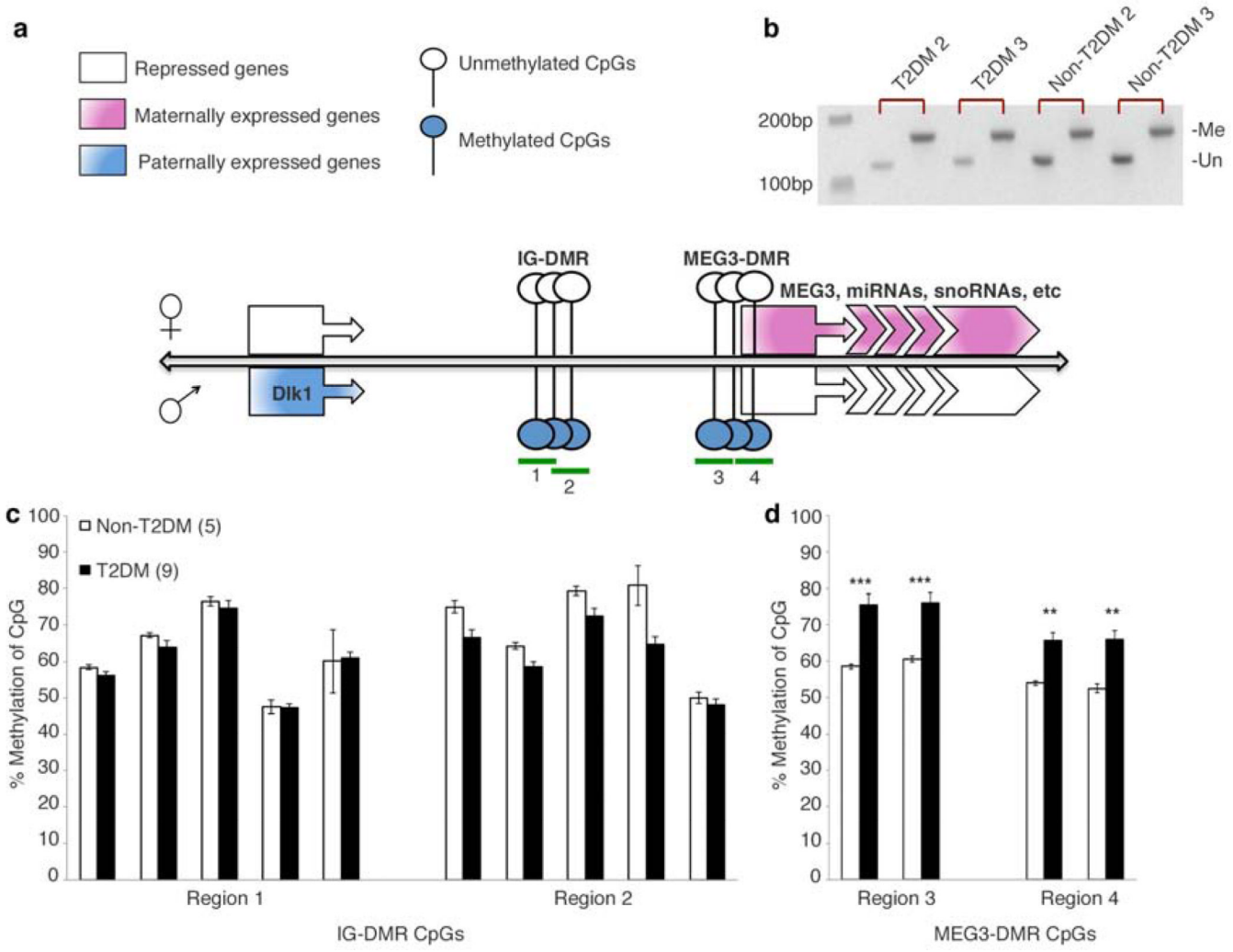

Fig 3. Increased methylation of the MEG3-Differentially methylated region (DMR) in T2DM islets

(a) Schematic representation of DLK1-MEG3 locus DMRs with allele specific gene expression depicted. Regions analyzed for Fig 3c and d are marked as green bars. (b) Methylation-specific PCR for MEG3 promoter methylation in two T2DM and two non-diabetic donors' islets. The methylated band (Me) is 160 bp, the unmethylated band (Un) is 120 bp. Percent methylation was determined for multiple CpGs in the (c) IG-DMR and (d) MEG3-DMR in 5 non-diabetic and 9 T2DM donors' islets by pyrosequencing of bisulfite converted DNA. Each bar represents an individual CpG, and the regions refer back to schema in part (a). p-value calculated using Student's T-test. **p-value<0.005, ***p-value<0.001. +/− SEM. See also Figure S3.

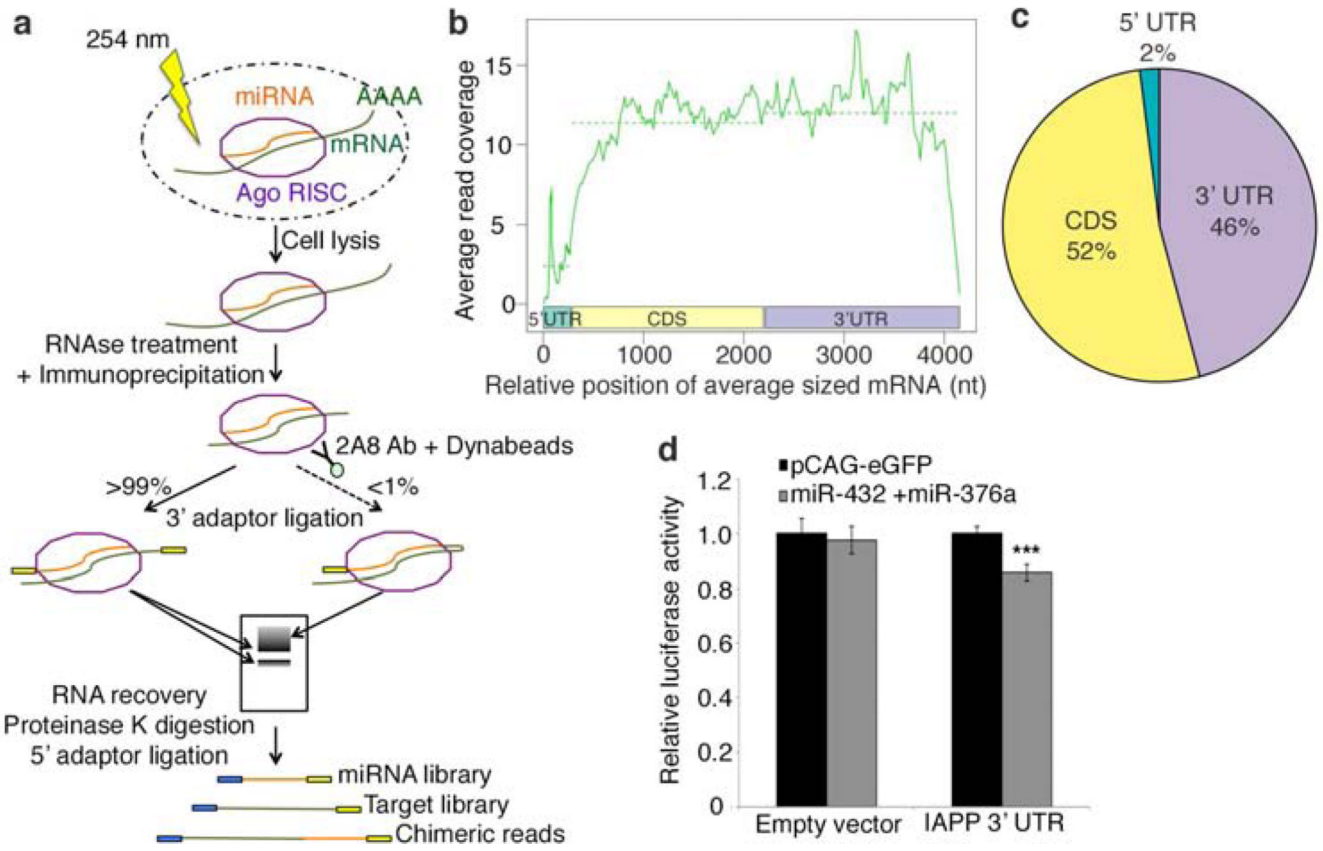

Fig 4. Identification of miRNA targets in human islets by HITS-CLIP

(a) Schema of HITS-CLIP procedure and chimeric reads ligation. (b) Average read coverage of all HITS-CLIP target mRNA fragments over a standardized mRNA. (c) Argonaute footprint distribution across target library mRNAs in human islets. (d) Targeting of human IAPP mRNA by miR-432 and 376a was validated by luciferase reporter assays. Vectors with or without the 3'UTR of IAPP were co-transfected with either empty pCAG-eGFP vector or miR-432 and -376a. Error bars indicate mean +/− SEM. ***p-value calculated using Student's t-test. $p = 1.8 \times 10^{-5}$. See Tables S4 and S5.

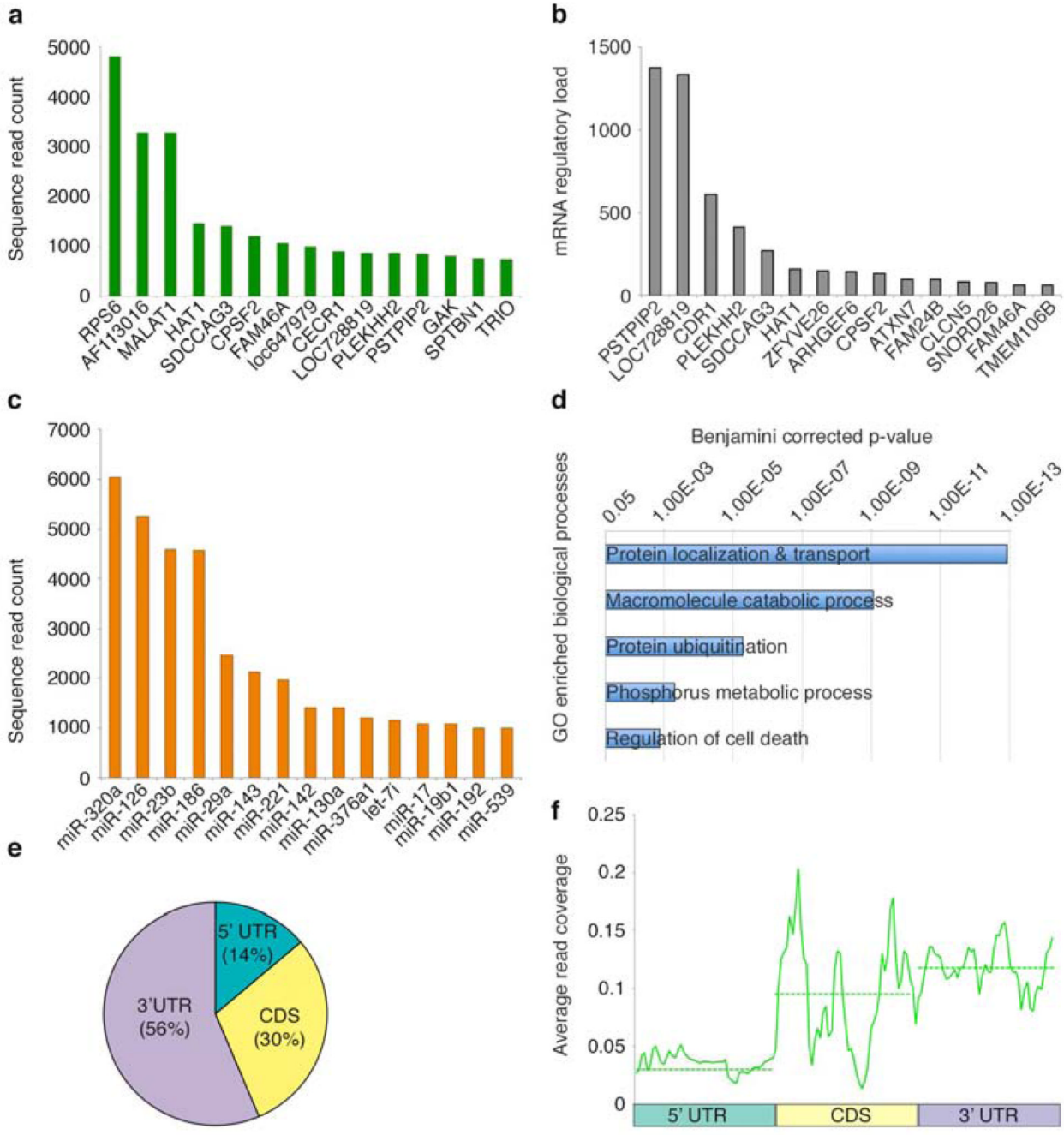

Fig 5. Determination of direct miRNA:mRNA targeting relationship from chimeric reads

Deep sequencing of our Argonaute HITS-CLIP library identified thousands of chimeric reads, consisting of a mature miRNA and a target mRNA fragment. (a) The fifteen most abundant mRNAs found in chimeric reads in human islets. (b) The fifteen most highly miRNA-regulated mRNAs in chimeric reads. The regulatory load ratio is the relative Ago-associated mRNA fraction of the chimeric reads, defined as the ratio of their sequence counts to their normalized abundance in human islets (c) The fifteen most abundant miRNAs found in chimeric reads in human islets. (d) Significantly enriched gene ontology biological processes in targets of human islet miRNAs. (e) Pie-chart representation of distribution of mRNA regions found in chimeras with miRNAs. (f) Average read coverage

of chimeric mRNA fragments across an mRNA divided into 150 equal bins. See Figure S4 and Table S6.

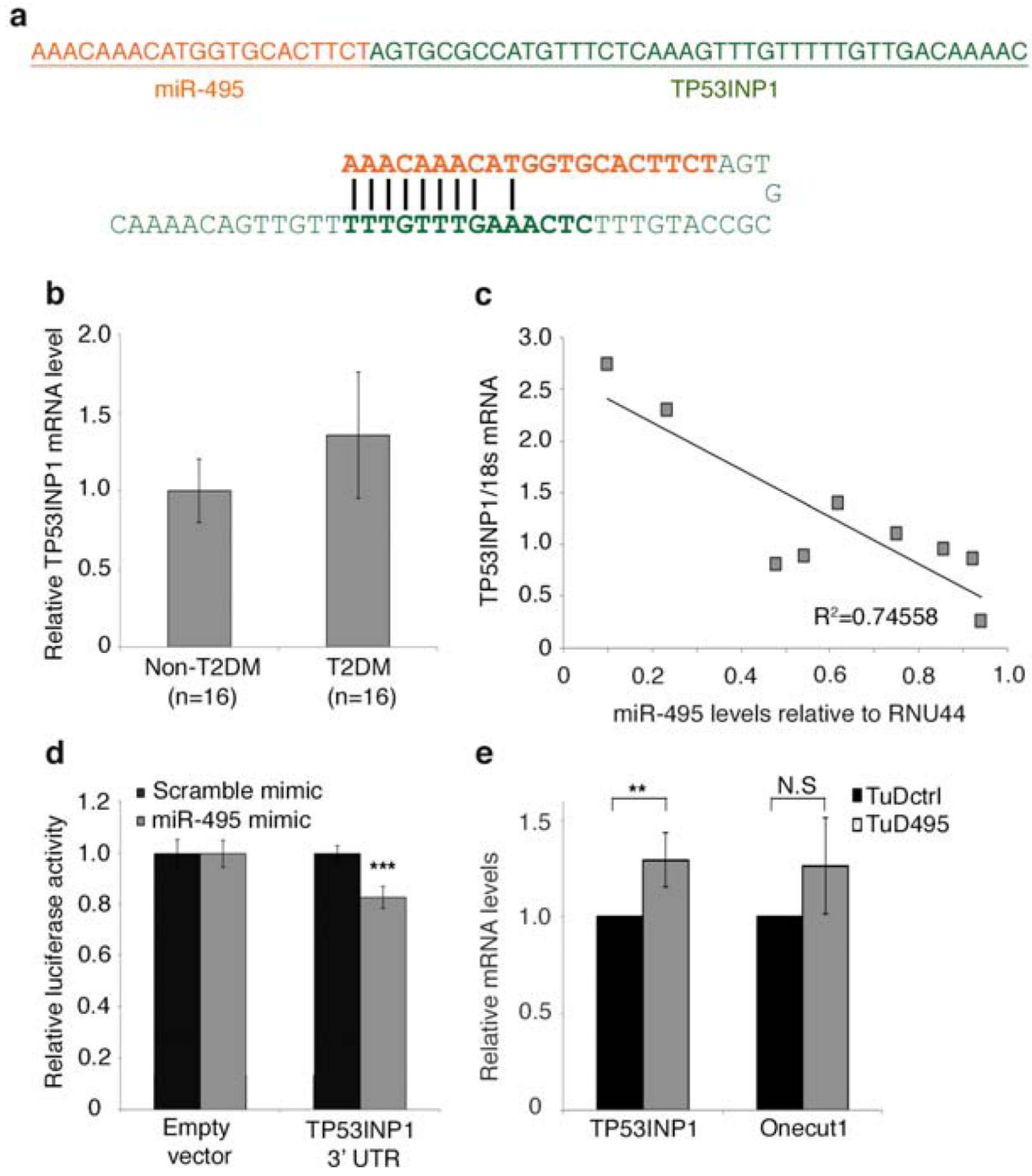

Fig 6. Validation of the miR-495:TP53INP1 targeting relationship

A β-cell apoptotic factor, TP53INP1 is regulated by miR-495. (a) The sequence of the miR-495 (orange) and TP53INP1 3'UTR (green) chimera. Folded confirmation with base pairing between the miRNA and 3'UTR is indicated below. (b) Relative levels of TP53INP1 mRNA between T2DM and non-T2DM islet samples. Error bars indicate mean +/− SEM. (c) Anti-correlation between normalized TP53INP1 and miR-495 in nine T2DM islet donor samples. (d) Targeting of human TP53INP1 mRNA by miR-495 was validated by luciferase reporter assays. Vectors with or without the 3'UTR of TP53INP1 were co-transfected with either scramble or miR-495 mimics. Error bars indicate mean +/− SEM. p-value calculated using Student's t-test. ***p-value = 1.94×10^{-5}. (e) Relative mRNA levels of TP53INP1

and Onecut1 (normalized to the average of HPRT and beta actin transcript levels) in human islets transduced with lentivirus encoding tough decoy constructs for either scramble sequence (TuDctrl) or miRNA-495 (TuD495). Error bars indicate +/− SEM. **p-value = 0.0076, n=3.

Table 1

Islet donor information

Characteristics of T2DM and non-T2D islet donors used in small RNA-sequencing screen are listed. Related to Fig 1 and Table S1.

Donor ID	Age	Gender	Blood Type	BMI	Race	Cause of Death
Non T2DM 1	33	Female	A+	31.1	African American	Anoxia, cardiovascular (CVA)
Non T2DM 2	22	Female	O+	24.9	African American	Head trauma
Non T2DM 3	51	Male	O+	26.1	Hispanic/Latino	Head trauma
T2DM 1	61	Female	A−	29.6	Caucasian	Anoxia, CVA
T2DM 2	45	Male	B+	37	Caucasian	Anoxia, CVA
T2DM 3	53	Male	O+	38.4	Caucasian	CVA
T2DM 4	54	Female	A	21.6	Hispanic/Latino	CVA