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Epigenetic Regulation of the Dlk1-Meg3 Imprinted Locus in Human Islets

Abstract
Type 2 diabetes mellitus (T2DM) is a complex metabolic disease characterized by inadequate insulin secretion by the pancreatic β-cell in response to increased blood glucose levels. Despite compelling evidence that T2DM has a high rate of familial aggregation, known genetic risk variants account for less than 10% of the observed heritability. Consequently, post-transcriptional regulators of gene expression, including microRNAs and other noncoding RNAs, have been implicated in the etiology of T2DM, in part due to their ability to simultaneously regulate the expression of hundreds of targets.

To determine if microRNAs are involved in the pathogenesis of human T2DM, I sequenced the small RNAs of human islets from diabetic and non-diabetic organ donors. From this screen, I identified the maternally-expressed genes in the imprinted DLK1-MEG3 locus as highly- and specifically-expressed in human β-cells, but repressed in T2DM islets. Repression of this noncoding transcript was strongly correlated with hyper-methylation of the promoter that drives transcription of all the maternal noncoding RNAs including the long noncoding RNA MEG3, several microRNAs and snoRNAs. Additionally, I identified disease-relevant targets of DLK1-MEG3 microRNAs in vivo using HITS-CLIP, a technique to detect targets of RNA binding proteins. My results provide strong evidence for a role of microRNAs and epigenetic modifications, such as DNA methylation, in the pathogenesis of T2DM. In addition, my data set catalogs human islet microRNAs relevant to human T2DM pathogenesis and characterizes their target transcriptomes.

Despite being associated with T2DM and several other diseases, very little is known about the regulation of imprinting of the MEG3-DLK1 locus. Hence, I interrogated a newly described enhancer in this locus, as enhancers are known mediators of mono-allelic expression at other imprinted loci. I discovered allele-specific binding of this enhancer by critical islet transcription factors, including FOXA2 in human islets. In addition, I mapped long-range interactions of this enhancer in human islets using 4C-Seq. Overall, my findings provide novel insights into the regulation of an imprinted locus critical to β-cell health and function.

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EPIGENETIC REGULATION OF THE *DLK1-MEG3* IMPRINTED LOCUS IN HUMAN ISLETS

Vasumathi Kameswaran

A DISSERTATION

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Dedicated to my grandmother, Dr. Lalitha Kameswaran.

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It would be remiss of me to ignore my sources of caffeine. Gia Pronto, La Colombe and Starbucks, I owe you guys!

ABSTRACT

EPIGENETIC REGULATION OF THE *DLK1-MEG3* IMPRINTED LOCUS IN HUMAN ISLETS

Vasumathi Kameswaran

Klaus H. Kaestner

Type 2 diabetes mellitus (T2DM) is a complex metabolic disease characterized by inadequate insulin secretion by the pancreatic β-cell in response to increased blood glucose levels. Despite compelling evidence that T2DM has a high rate of familial aggregation, known genetic risk variants account for less than 10% of the observed heritability. Consequently, post-transcriptional regulators of gene expression, including microRNAs and other noncoding RNAs, have been implicated in the etiology of T2DM, in part due to their ability to simultaneously regulate the expression of hundreds of targets.

To determine if microRNAs are involved in the pathogenesis of human T2DM, I sequenced the small RNAs of human islets from diabetic and non-diabetic organ donors. From this screen, I identified the maternally-expressed genes in the imprinted *DLK1-MEG3* locus as highly- and specifically-expressed in human β-cells, but repressed in T2DM islets. Repression of this noncoding transcript was strongly correlated with hyper-methylation of the promoter that drives transcription of all the maternal noncoding RNAs including the long noncoding RNA *MEG3*, several microRNAs and snoRNAs. Additionally, I identified disease-relevant targets of *DLK1-MEG3* microRNAs *in vivo* using HITS-CLIP, a technique to detect targets of RNA binding proteins. My results provide strong evidence for a role of microRNAs and epigenetic modifications, such as DNA methylation, in the pathogenesis of T2DM. In addition, my data set catalogs human islet microRNAs relevant to human T2DM pathogenesis and characterizes their target transcriptomes.

Despite being associated with T2DM and several other diseases, very little is known

about the regulation of imprinting of the *MEG3-DLK1* locus. Hence, I interrogated a newly described enhancer in this locus, as enhancers are known mediators of mono-allelic expression at other imprinted loci. I discovered allele-specific binding of this enhancer by critical islet transcription factors, including FOXA2 in human islets. In addition, I mapped long-range interactions of this enhancer in human islets using 4C-Seq. Overall, my findings provide novel insights into the regulation of an imprinted locus critical to β-cell health and function.

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Chapter 1

Introduction

Diabetes Mellitus

Glucose homeostasis is achieved through the coordinate action of hormones secreted by the islets of Langerhans, the endocrine compartment of the pancreas. Islets consist of four principal secretory endocrine cells – α-, β-, δ- and pancreatic polypeptide (PP) producing cells (Bonner-Weir and O'Brien 2008). Insulin secreted by the β-cells facilitates the uptake of glucose from the blood stream by insulin-responsive tissues while also repressing glucose production and ketogenesis from the liver. In contrast, glucagon, a product of the α-cell, is secreted during conditions of low blood glucose primarily to stimulate hepatic glucose output. The actions of both α- and β-cells are regulated in a paracrine manner by the somatostatin-producing δ-cells. Human and non-human primate islets display high variability in their cellular composition and architecture. A typical human islet consists of approximately 54% β-cells, 34% α-cells and 10% δ-cells, with the different cell types distributed heterogeneously throughout the islet (Brissova et al. 2005; Cabrera et al. 2006).

Diabetes mellitus is a complex metabolic disorder characterized by high blood glucose levels as a result of inadequate insulin production or action. The autoimmune destruction of the insulin-producing β-cells results in Type 1 diabetes (T1DM). T1DM accounts for a small fraction of diabetes cases, but this form of the disease manifests at a young age and affected individuals are dependent on insulin therapy for life, severely compromising their lifestyle. In Type 2 diabetes (T2DM), the more common form of the disease, the defect in glucose metabolism is the result of decreased sensitivity of peripheral tissues to insulin action, accompanied by failure of β-cells to compensate for the increased metabolic demand (Ashcroft and Rorsman 2012). The International Diabetes Federation estimated that in 2014, more than 387 million people worldwide were affected by this disease, with an anticipated increase of 205 million people by the year 2035 (https://www.idf.org/diabetesatlas). Chronic hyperglycemia resulting from insulin deficiency is associated with increased risk for many long-term complications such as heart disease, renal failure, blindness, and peripheral neuropathy. Consequently, the diabetes pandemic not only

represents a global healthcare issue but also an economic burden, with diabetes-related expenditures accounting for 612 billion dollars worldwide in the year 2014.

Although T2DM has traditionally been considered to be solely a disease of insulin resistance, it is becoming increasingly clear that the compensatory response of β-cells influences diabetes etiology (Prentki and Nolan 2006; Ashcroft and Rorsman 2012). A longitudinal study conducted in Pima Indians of Arizona, a population of individuals with a high propensity for non-autoimmune diabetes, demonstrated that impaired insulin secretion was an early determinant of diabetes progression (Weyer et al. 1999). Similarly, β-cell mass was lower in islets from T2DM donors compared to body mass index (BMI)-matched non-diabetic donor islets, while β-cell mass was increased in islets obtained from non-diabetic obese individuals relative to lean controls (Butler, Janson, Bonner-Weir, et al. 2003). These studies and others highlight the importance of an adaptive β-cell response to increased metabolic demand in the etiology of T2DM. Moreover, there is increasing evidence that inherent defects of the β-cells may increase their susceptibility to autoimmune destruction in T1DM (Soleimanpour and Stoffers 2013). Thus, understanding the molecular basis of β-cell dysfunction is imperative to improve diabetes treatment strategies.

Heritable determinants of T2DM risk

The high familial predisposition for developing T2DM has long been recognized, and is supported by the higher concordance rate for T2DM among siblings and monozygotic twins compared to general population risk (Medici et al. 1999; Hemminki et al. 2010). In fact, among Caucasian individuals, the risk for developing T2DM is 3.5-fold higher for offspring of a single diabetic parent, and 6-fold higher when both parents are diabetic, compared to offspring without parental diabetes (Meigs, Cupples, and Wilson 2000). As a result, efforts have been directed towards identifying the underlying causal gene mutations and variants, with the hope that this information will improve diagnostic and preventative strategies. These gene discovery efforts have been particularly effective in the identification of causal genes for maturity-onset diabetes of the young (MODY), a rare monogenic form of non-autoimmune diabetes characterized by

autosomal dominant transmission (Tattersall and Fajans 1975). MODY is estimated to constitute between 1 and 3% of diabetes cases. Several genes associated with MODY have now been identified.

Efforts to identify causal genes for more common forms of diabetes have been only partially successful in explaining the heritability of T2DM. Early candidate gene-association studies suffered from poor reproducibility and were statistically under-powered (Frayling 2007). Nonetheless, common coding variants in *PPARG* (Altshuler et al. 2000) and *KCNJ11* (Gloyn et al. 2003) were found to confer a modest risk for T2DM, and have since been confirmed as bona fide risk alleles. The most recent wave of gene discovery has come in the form of unbiased large-scale studies relating of common sequence polymorphisms with disease phenotypes, so-called genome-wide association studies (GWAS). Using this approach, more than a hundred genes are have been associated with T2DM and related traits (Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University, and Novartis Institutes of BioMedical Research et al. 2007; Scott et al. 2007; Voight et al. 2010). Despite the high rate of gene prediction from such studies, there are several outstanding challenges in the interpretation of these results. The risk variants identified through these studies generally serve as molecular markers of the haplotype, and in most cases, the causal sequences themselves are yet to be determined. The presence of multiple genes within a co-inherited haplotype block, and the non-coding landscape of many candidate variants confounds the ability to associate a biological function to these sequences (Mccarthy 2010). Most importantly, the risk SNPs identified so far have very low effect sizes and together account for only approximately 10% of the heritable transmission of T2DM risk (Frayling 2007; Mccarthy 2010). Overall, these observations have urged investigations into alternative explanations for this unresolved heritable component in T2DM etiology. Some of these molecular candidates include microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and epigenetic regulators (Bramswig and Kaestner 2012).

MicroRNAs in β-cell function and T2DM pathogenesis

MiRNAs have emerged as essential regulators of several developmental processes, due to their evolutionary conservation, tissue- and stage-specific expression, and ability to fine-tune expression of multiple targets simultaneously (Ebert and Sharp 2012). Similar to the interest in mapping the transcription factor networks critical to pancreas development and adult function (Pasquali et al. 2014), the potential contribution of miRNAs to these processes has also been under intense scrutiny. Over the past decade, a large body of evidence has collectively implicated multiple miRNAs in β-cell function, and consequently, diabetes pathogenesis.

MicroRNA biogenesis

MicroRNAs are small ~22nt RNAs that post-transcriptionally regulate gene expression through multiple mechanisms. This class of RNAs was first identified through the discovery of a group of genes that are critical to *C.elegans* larval development but do not encode proteins (R. C. Lee, Feinbaum, and Ambros 1993; Reinhart et al. 2000). The recognition that these small RNAs were complimentary to the 3'UTR of specific mRNA transcripts involved in stage-specific larval development (R. C. Lee, Feinbaum, and Ambros 1993; Reinhart et al. 2000) and that this RNA-based temporal gene regulation was conserved across multiple species (Pasquinelli et al. 2000) opened an entire field dedicated to the study of these small RNAs, subsequently named microRNAs (miRNAs) (Lagos-Quintana et al. 2001; Lau et al. 2001; R. C. Lee and Ambros 2001). Initial data mining studies identified common features of these miRNAs across multiple species, including the fact that they are broadly distributed across the genome. MicroRNAs can exist as independent transcriptional units, or be contained within introns of protein-coding genes. Some miRNAs were also found to be organized in genomic clusters, and are likely transcribed as a single poly-cistronic primary transcript (Lagos-Quintana et al. 2001; Lau et al. 2001; Mourelatos et al. 2002; Y. Lee et al. 2002; Seitz et al. 2004).

The complex processing steps that result in miRNA biogenesis are now well defined. Primary miRNA transcripts (pri-miRNAs) are mainly transcribed by RNA polymerase II (Y. Lee et

al. 2004) (Y. Lee et al. 2002) (in some cases, RNA polymerase III (Borchert, Lanier, and Davidson 2006)) and are subsequently cleaved by the RNAseIII endonuclease Drosha (Y. Lee et al. 2003) to generate conserved ~70bp stem-loop structures (pre-miRNA) (Lagos-Quintana et al. 2001; Lau et al. 2001; Mourelatos et al. 2002). The pre-miRNAs are exported from the nucleus and processed further by another RNAseIII enzyme, Dicer (E. Bernstein et al. 2001; Hutvágner et al. 2001) to produce the mature miRNA, which guides the Argonaute protein of the miRNA Induced Silencing Complex (RISC) to its target mRNA (Hutvágner and Zamore 2002; Mourelatos et al. 2002; Peters and Meister 2007). Notably, some miRNAs are refractory to these processing steps, such as the *dicer*-independent miR-451 (Cifuentes et al. 2010). Besides Argonaute, the RISC complex consists of other co-effectors that catalyze target repression, such as the protein GW182 (Eulalio, Tritschler, and Izaurralde 2009). Together, the RISC complex mediates target repression by several mechanisms, including blocking cap-dependent translation initiation and ribosomal loading, as well as recruitment of mRNA deadenylation and destabilization complexes (Chendrimada et al. 2007; Kiriakidou et al. 2007; Eulalio, Huntzinger, and Izaurralde 2008; Fabian et al. 2011; Filipowicz, Bhattacharyya, and Sonenberg 2008). Studies assaying the kinetics of these closely linked processes have established that miRNA-mediated translational repression precedes mRNA deadenylation and transcript destabilization (Bazzini, Lee, and Giraldez 2012; Djuranovic, Nahvi, and Green 2012; Selbach et al. 2008).

MicroRNA target prediction

The initial discovery that miRNAs lin-4 and let-7 were complimentary to the 3’ UTR of their target mRNAs and that they affected target protein rather than mRNA levels (R. C. Lee, Feinbaum, and Ambros 1993; Reinhart et al. 2000) suggested that miRNAs function to regulate gene expression post-transcriptionally in a manner that depended on sequence complementarity (Baek et al. 2008; Selbach et al. 2008). Since then, several computational algorithms have been developed to predict targets of miRNAs genome-wide. While each program offers subtle differences in prediction outcomes, they are largely based on similar assumptions, such as

complementarity of the “seed” sequence (position 2-8 of the miRNA 5’ region), evolutionary conservation of the target site, and combinatorial regulation of a given target by multiple miRNAs, or the presence of several binding sites for a single miRNA in the same mRNA species (Lewis et al. 2003; Kiriakidou et al. 2004). These algorithms provide a useful platform for identifying candidate targets of miRNAs. While such programs help select mRNA targets that are very likely to be regulated by miRNAs, they do not always take into consideration factors such as temporal and tissue-specific expression of the miRNAs and mRNAs being paired. Additionally, many computational programs overlook functional interactions that are exceptions to the generally accepted rules of miRNA-target pairing. This includes the inclusion of wobble (G:U) base pairing in the seed sequence, and less stringent seed sequence binding coupled with compensatory complementarity of the 3’ end of the miRNA to the target (Brennecke et al. 2005). On the other hand, inclusion of these non-canonical binding parameters into the algorithms increases the likelihood of spurious target predictions. Thus, bioinformatics approaches often have a high rate of false positive and false negative miRNA:mRNA targeting relationships, depending on the stringency of parameters being used, making the identification of biologically relevant miRNA targets solely relying on computational tools challenging.

As a way to circumvent such issues, the Darnell lab developed high-throughput sequencing of RNAs isolated by cross-linking and immunoprecipitation (HITS-CLIP) of Argonaute, a key component of the RISC (Chi et al. 2009). This method relies on ultraviolet irradiation-induced cross-linking of Argonaute to the RNAs in its proximity, thus allowing for immunprecipitation of both miRNAs and mRNAs that are bound by Argonaute *in vivo* under stringent conditions. Thus, the technique allows for the simultaneous isolation of Argonaute-bound miRNAs and mRNA fragments that are relevant to the tissue of interest. Although HITS-CLIP still relies on computational algorithms to match the miRNAs to their cognate targets, it drastically improves false-positive and false-negative prediction rates relative to traditional bioinformatics approaches by reducing the computational search space (Chi et al. 2009).

Additionally, HITS-CLIP has facilitated the identification of alternative miRNA binding

patterns (Chi, Hannon, and Darnell 2012). This protocol has been further modified to incorporate photoactivateable nucleoside analogs, such as 4-thiouridine (PAR-CLIP) to map the Argonaute footprint with finer resolution (Kirino and Mourelatos 2008; Hafner et al. 2010). An alternative approach to identify targets of miRNAs *in vivo* termed RISCtrap employs a dominant negative GW182 mutant protein to stabilize miRNA targets that are typically subjected to deadenylation and hence under-represented in CLIP-based techniques (Cambronne et al. 2012). This GW182 mutant can associate with Argonaute through its GW-rich N-terminus, and hence both the miRNA and its mRNA target are loaded onto it, but it can no longer recruit cytoplasmic deadenylation complexes through its mutated C-terminal domain (Eulalio et al. 2009). While PAR-CLIP and RISCtrap provide several pertinent improvements to the original HITS-CLIP protocol, their applicability is limited to *in vitro* assays. A more recent adaptation of HITS-CLIP, CLASH, partially overcomes the need for bioinformatic matching of miRNAs to their targets by facilitating the intramolecular ligation of Argonaute-bound miRNAs to their target mRNAs (Helwak et al. 2013). Although these hybrids only represent a small fraction of the sequenced RNAs (2%), they are an invaluable source of miRNA targeting principles. Indeed, *in vivo* target identification methods such as HITS-CLIP and CLASH have revealed that miRNA recognition elements are present not only in the 3'UTR but also the 5'UTR and coding regions of the mRNA. Additionally, non-canonical seed interactions occurred more frequently than perfect base-pairing. Overall, although our understanding of miRNA targeting principles has drastically improved over the past decade, we are still limited in our technical ability to identify functional targets of miRNAs, warranting thorough validations of such predicted targeting events.

The role of miRNAs in islet-development and function

A common strategy to examine the general role of miRNAs in developmental processes is to delete the obligatory miRNA-processing enzyme, *Dicer1* in a specific tissue or cell lineage. Germline *Dicer1* deletion results in embryonic lethality due to a loss of stem cells (E. Bernstein et al. 2003),. However, *Dicer1* hypomorphic mice with a 20% loss of *Dicer1* expression are viable

and histologically normal in all tissues except for the pancreas (Morita et al. 2009). These mice exhibit abnormal endocrine cell distribution and size as well as ectopic expression of endocrine markers in ductal epithelial cells.

Conditional deletions of *Dicer1* using the Cre/LoxP technology at various developmental stages have refined the role of miRNAs in pancreas development, and have collectively revealed that miRNAs are essential to normal endocrine cell development and adult function. The first indication of a role for miRNAs in pancreatic development came from the inactivation of *Dicer1* in early pancreatic progenitor cells expressing *Pdx1* (Lynn et al. 2007). These mice die shortly after birth and display defects in all pancreatic lineages, although the loss of endocrine cells, particularly β- and δ- cells, was the predominant phenotype. The authors also deleted *Dicer1* in endocrine progenitor or mature β-cells using Cre recombinase under the control of Neurogenin3 (Ngn3) or the rat insulin promoter (RIP) respectively. As they did not observe altered islet morphology, the authors concluded that miRNAs primarily function in early pancreatic progenitor cells rather than in mature endocrine cell maintenance. However, islet functionality was not assessed in this study, and phenotypes that manifest later were likely to be missed by this preliminary characterization.

Subsequent studies in mice with *Dicer1* deletion in endocrine precursor cells using *Ngn3*-Cre revealed that these mice develop the endocrine cell types appropriately but develop diabetes within two weeks of birth (Kanji, Martin, and Bhushan 2013). These mice exhibit a severe loss of endocrine cell mass and hormone expression. The down-regulation of insulin expression is independent of its known transcription regulators such as *Pdx1*, *Nkx6.1* and *MafA*. Interestingly, following cells lacking *Dicer1* using a genetic lineage tracing system, the authors demonstrate that the *Dicer1*-ablated islet cells are more susceptible to apoptosis than control cells.

Contrary to the observations of Lynn *et al*, two groups reported that inactivation of *Dicer1* in mid-gestation mouse β-cells using the RIP-Cre transgene results in hyperglycemia and diabetes by adulthood (Kalis et al. 2011; Mandelbaum et al. 2012). In this model, both insulin synthesis and β-cell mass were severely compromised. However, unlike in the case of *Dicer1*

deletion in the Ngn3-expressing cells (Kanji, Martin, and Bhushan 2013), there was no observable difference in apoptosis between mutant and control β-cells. The conditional ablation of *Dicer1* in adult β-cells using an inducible RIP-Cre ER transgene resulted in the mice developing severe hyperglycemia and overt diabetes within two weeks following gene ablation (Melkman-Zehavi et al. 2011). Contrary to the studies discussed above, β-cell mass was unchanged in *Dicer1*-null adult β-cells, suggesting that miRNAs are likely to regulate islet architecture only at early developmental stages. Insulin expression and content was decreased due to the aberrant activation of repressors of insulin transcription. Similar to the observations of Kanji *et al*, transcript levels of transcriptional activators of insulin expression, such as *Pdx1* and *MafA,* were unchanged in *Dicer1* mutant cells. In summary, results from *Dicer1* knockout studies in the pancreas indicate that miRNAs are essential regulators of endocrine cell development, cellular composition and β-cell function.

Islet miRNAs

One of the best characterized and most abundant miRNAs in the murine and humanpancreatic islet is miR-375 (Poy et al. 2004). This miRNA is very highly expressed in the developing mouse (Lynn et al. 2007) and human (M. V. Joglekar, Joglekar, and Hardikar 2009) pancreas, as well as in mature mouse islet cells (Poy et al. 2004). Through *in vitro* characterizations in mouse β-cell lines, it was determined that miR-375 is a repressor of glucose stimulated insulin secretion, acting primarily to inhibit exocytosis through its target, myotrophin. In a subsequent study, it was reported that miR-375 negatively regulates PDK1, a critical component of the PI3K/PKB signaling cascade (Ouaamari et al. 2008). Using both gain- and loss-of-function approaches in rat insulinoma cells, the authors demonstrate that miR-375 negatively regulates PDK1 protein levels. This results in reduced PDK1-mediated insulin signaling and ultimately affects β-cell viability, proliferation and insulin transcription. Overall, these studies proposed a role for miR-375 as a negative regulator of insulin signaling and secretion.

Rather surprisingly, mice lacking miR-375 were found to be hyperglycemic, which was primarily attributed to increased α-cell mass and function (Poy et al. 2009). The mechanism by which miR-375 contributes to hyperglucagonemia is yet to be determined. However, miR-375 null mice exhibit a modest decrease in β-cell mass and miR-375 expression is increased in leptin-deficient *ob/ob* mice, a model for insulin resistance and increased β-cell mass. Lack of miR-375 resulted in the inability of these islets to compensate for insulin resistance, suggesting that miR-375 plays an important role in the β-cell compensatory response under conditions of increased metabolic stress. MiR-375 expression was also down-regulated in islets from diabetic Goto-Kakizaki (GK) rats (Ouaamari et al. 2008). These results support a role for miR-375 in islet function and glucose homeostasis, although the mechanism by which it functions remains to be elucidated and the contradictory results have yet to be reconciled.

Another highly expressed miRNA in rodent and human islets is miR-7 (Bravo-Egana et al. 2008). The expression of this miRNA is restricted to the endocrine compartment of fetal, neonatal and adult human pancreata (M. V. Joglekar, Joglekar, and Hardikar 2009; Correa-Medina et al. 2009; Bravo-Egana et al. 2008). MiR-7 has been demonstrated to be a negative regulator of pancreatic β-cell proliferation (Wang et al. 2013), and it acts by targeting multiple components of the mTOR signaling pathway. In this study, decreasing endogenous levels of miR-7a, the prominent form of miR-7 in mouse β-cells, resulted in a modest increase in cell proliferation. Using overexpression and miR-7a suppression approaches, the authors demonstrate that miR-7a directly modulates the activity of luciferase reporter constructs containing the conserved 3'UTR region of each target. Suppression of endogenous miR-7a levels in cultured and primary mouse β-cells resulted in activation of the mTOR signaling cascade. Additionally, miR-7a repression resulted in increased proliferation of primary mouse and human β-cells, and this phenotype was dependent on mTOR signaling. Because of its importance in β-cells, mice with a conditional deletion of *Mir7a2*, the predominant miR-7a precursor transcript, were derived. These mice exhibited improved glucose tolerance as a result of enhanced insulin secretion (Latreille et al. 2014). Conversely, transgenic mice overexpressing *Mir7a2* were hyperglycemic and had impaired

insulin secretion. By assaying various aspects of the insulin secretory pathway, the authors determined that miR-7a negatively regulates insulin secretion by modulating the β-cell exocytotic machinery. However, in the miR-7a characterization by Wang *et al*, no difference in insulin secretion was observed. The discrepancy in phenotype may be explained by the different development had been points at which miR-7a was inactivated in these studies, as well as the different model systems used. Despite this difference, the results of Letreille *et al* do not preclude those of Wang *et al*. In fact, the inverse correlation between miR-7a expression levels and β-cell compensation in genetic mouse models of obesity (*ob/ob*) and diabetes (*db/db*) noted by Letreille *et al* could be the result of altered β-cell proliferation. Together, these investigations highlight the importance of miR-7a as a negative regulator of β-cell function.

Overall, studies in rodent and *in vitro* models of diabetes have irrefutably established miRNAs as key regulators of glucose homeostasis (Guay et al. 2012). However, these models do not fully recapitulate the complexity of human T2DM pathogenesis, and so it is essential to characterize the miRNA transcriptome of primary human islets. One such study profiled the expression of miRNAs from human primary islets, liver and skeletal muscle - tissues relevant to glucose metabolism (Bolmeson et al. 2011). This study resulted in the identification of a subset of islet-enriched miRNAs including, miR-375, miR-127, and miR-184. Following this, comparative expression profiling of miRNAs in β-cells relative to whole islets (van de Bunt et al. 2013) and α-cells (Klein et al. 2013) were reported. To explore the possible role of miRNAs in the genetic predisposition of T2DM pathogenesis, van de Bunt *et al* compared islet-enriched miRNA precursor sequences as well predicted targets of these miRNAs with risk variants associated with T2DM (van de Bunt et al. 2013). This analysis revealed that predicted targets of islet-expressed miRNAs overlapped with several SNPs associated with T2DM risk by GWAS. Remarkably, a risk variant in the 3'UTR of *SLC30A8*, overlapped with predicted target sites for six islet-enriched miRNAs. Surprisingly, the comparative analysis of miRNA expression between FACS-purified α- and β-cells identified only 7 miRNAs as preferentially expressed in α-cells compared to 134 in β-cells (Klein et al. 2013). In fact, the 3'UTRs of α-cell specific transcription factors *cMaf* and *Zfpm2*

were highly enriched for recognition sites of β-cell specific miRNAs, suggesting that miRNAs play a role in the regulation of lineage-specific gene expression in human islets.

In summary, this growing body of literature strongly suggests that miRNAs are critical regulators of glucose homeostasis and that their mis-regulation may be detrimental to β-cell function (figure 1.1). These findings motivated our studies to determine if the miRNA transcriptome is dysregulated in islets of human T2DM patients, which will be discussed in Chapter 3.

Long non-coding RNAs

Recent technological advances in the field of genome sequencing have paved the way for a new appreciation of non-coding RNAs in gene regulation. Ultra high-throughput transcriptome analyses have revealed that the vast majority of the genome is transcribed, with two-thirds of the human genome covered by processed transcripts, of which only a small fraction (<2%) is translated into proteins (Djebali et al. 2012). The identification of several common genomic and functional features of these untranslated RNAs has led to their categorization into various classes of non-coding RNAs. One such class that has been the focus of extensive research is that of long non-coding RNAs (lncRNAs). As novel mechanisms of lncRNA-mediated gene regulation come to light, their involvement in the regulation of complex metabolic pathways are being interrogated.

LncRNA description and general functions

LncRNAs are defined as transcripts longer than 200bp that lack protein-coding potential (Derrien et al. 2012; Fatica and Bozzoni 2014; Batista and Chang 2013; Guttman et al. 2009). Like mRNAs, lncRNAs typically have multiple exons, are processed using canonical splice sites, and may exist as several isoforms (Derrien et al. 2012; Ponjavic, Ponting, and Lunter 2007; Cabili et al. 2011). In contrast to mRNAs, lncRNAs preferentially display nuclear localization, consistent with their proposed function in chromatin organization and regulation of gene expression (Derrien

et al. 2012; Zhao et al. 2010; Khalil et al. 2009; Rinn and Chang 2012; Fatica and Bozzoni 2014; Guttman and Rinn 2012). Finally, despite their overall lower expression levels, lncRNAs exhibit a higher degree of tissue specificity compared to average protein-coding genes (Cabili et al. 2011; Mercer et al. 2008; Derrien et al. 2012; Fatica and Bozzoni 2014; Batista and Chang 2013).

Similar to protein-coding genes, lncRNA-encoding genes are marked by chromatin signatures typical of active transcription in the cell types where they are expressed, consisting of H3K4me3 (trimethylated lysine 4 in histone H3) at the promoter, followed by H3K36me3 along the transcribed regions (so-called "K4-K36 domains") (Guttman et al. 2009; Cabili et al. 2011; Khalil et al. 2009; Rinn and Chang 2012; Guttman and Rinn 2012). While lncRNA exons display weaker evolutionary conservation than those of protein-coding genes, there is evidence of positive selection for a subset of lncRNAs, which may be driven by constraints to maintain a specific secondary structure required for functional interactions with their chromatin targets (Derrien et al. 2012; Ponjavic, Ponting, and Lunter 2007; Guttman et al. 2009; Cabili et al. 2011; Ulitsky et al. 2011). In contrast, the promoters of lncRNAs are as highly conserved as those of protein-coding genes (Guttman et al. 2009; Derrien et al. 2012; Batista and Chang 2013; Ponjavic, Ponting, and Lunter 2007; Carninci et al. 2005).

Through numerous studies, several general principles of lncRNA function have emerged. LncRNAs have been shown to function both in *cis,* i.e. locally close to the site of their production, and in *trans*, i.e. at sites on other chromosomes. LncRNAs have been proposed to act as scaffolds for chromatin modifiers, as blockers of transcription, as antisense RNAs, microRNA sponges, protein decoys, and enhancers (Cech and Steitz 2014; Fatica and Bozzoni 2014). In fact, the act of transcription of a lncRNA itself can interfere with the regulatory function of a regulatory DNA sequence, as exemplified in yeast (Martens, Laprade, and Winston 2004) and in mammalian imprinting (Latos et al. 2012). As a result of their diverse functions in multiple tissues, mis-regulation of lncRNAs can lead to failure of normal development and, consequently, to disease. Mammalian chromatin modifiers such as the repressive *polycomb* complexes often lack their own specific DNA-binding domains, but instead contain RNA-binding elements. LncRNAs

can play critical roles in directing these repressive chromatin modifying complexes to their target regions (E. Bernstein and Allis 2005; Rinn et al. 2007; Zhao et al. 2010). These features suggest that lncRNAs and other non-coding RNA species may play an essential role in defining organismal complexity (Taft, Pheasant, and Mattick 2007; Mattick and Makunin 2006) and raise the possibility that lncRNAs and other non-coding RNAs may be exciting molecular candidates to account for the missing genetic risk in complex diseases such as diabetes (Medici et al. 1999; Hyttinen et al. 2003).

β-cell lncRNAs

The most comprehensive catalogue of human lncRNAs expressed in β-cells published thus far is that by Morán and colleagues (Morán et al. 2012). In this study, the authors profiled whole islet and FACS-sorted β-cells and identified 1,128 distinct transcripts that displayed many of the typical properties of lncRNAs, including “K4-K36” histone modification domains, lack of protein-coding potential, and non-uniform expression levels among tissues. Most notably, the lncRNAs identified were roughly five times more islet-specific compared to general protein-coding genes, and the vast majority had orthologous genes in the mouse genome. Ku and colleagues similarly characterized mouse islet- and β-cell-specific transcripts and identified 1,359 high-confidence lncRNAs with several of the afore-mentioned properties (Ku et al. 2012). Using high-throughput transcriptome analysis of sorted human islets, lncRNAs expressed preferentially in α-cells have also been identified (Bramswig et al. 2013).

Unlike some lncRNAs that are known to be critical to early stages of embryonic development (Guttman et al. 2011; Grote et al. 2013), the expression of a majority of islet lncRNAs appears to be restricted to differentiated, mature endocrine cells (Morán et al. 2012). The orthologous mouse lncRNAs (eg: *Mi-Lnc80*) exhibit similar cell- and stage-specific expression. The characteristics of these islet lncRNAs imply a role for these RNAs in mature β-cell function, although experimental evidence to support this notion is still lacking.

As previously noted, several risk variants for common forms of diabetes identified by GWAS do not change the protein-coding potential of known genes, suggesting that they might affect as yet unidentified regulatory elements (Mccarthy 2010). Using the computational tool MAGENTA to search for enrichment of genetic associations in a predefined set of genes (Segrè et al. 2010), Morán and colleagues determined that the islet lncRNA genes identified in their study were in fact highly enriched for risk alleles associated with T2DM and related phenotypes, further underscoring the need to interrogate the function of these RNAs in β-cell biology.

Overall, these studies highlight the fact that lncRNAs are a major component of the β-cell transcriptome that are cell-type-specific, developmentally regulated, and evolutionarily conserved with strong associations to disease risk. However, it still remains to be determined how these lncRNAs may contribute to β-cell function, and if their mis-regulation plays a directive role in diabetes. Future studies will also need to address the question whether the lncRNAs identified thus far act in *cis* (on neighboring islet protein-coding genes) or in *trans* to exert their function.

Epigenetics

Epigenetics refers to heritable changes in phenotype that do not result from a modification to the underlying genomic sequence. Covalent modifications to histone proteins and DNA sequence mediate these phenotypic changes and are collectively known as the "epigenome". During the course of mammalian development, the epigenome undergoes precise and dynamic changes that result in tissue-specific and temporal expression of genes, thus providing additional layers of organismal complexity not encoded in the genome. However, environmental factors can also trigger these stable phenotypic changes, altering the transcriptional outcome of the cell. Deciphering how the cell recognizes and interprets these environmental cues is a burgeoning field of particular interest to the diabetes community, as no definitive molecular link has been established between the escalating diabetes incidence and environmental factors such as diet and lifestyle.

Several experimental paradigms exemplify the likely gene-environment interaction in diabetes pathogenesis. Intrauterine growth retardation (IUGR) is a developmental complication resulting in low birth weight, and increased predisposition for T2DM in adulthood. In a rodent model of IUGR, caused by restricted hormone and nutrient supply to the fetus, loss of β-cell mass is accompanied by a progressive reduction in *Pdx1* mRNA and protein levels*.* This decrease in *Pdx1* gene activity was attributed to various epigenetic changes associated with decreased expression, including hypermethylation of the promoter and loss of activating modifications to histone H3 (Park et al. 2008). In humans as well, periconceptual exposure to malnutrition, as observed in individuals prenatally exposed to the Dutch Hunger famine, results in various adverse metabolic outcomes with underlying epigenetic changes (Heijmans et al. 2008). Thus, determining the epigenetic modifications induced by environmental stimuli is critical to understand the progression of metabolic disorders.

Histone modifications

The human genome consists of approximately 3 billion bases that are condensed into the cellular nucleus. DNA compaction is accomplished by winding of DNA into nucleosomes, which are comprised of 147 bp of DNA wrapped around a histone octomer, consisting of two histone H2A-H2B dimers and a H3-H4 tetramer. Aside from chromatin compaction, these histone proteins have been increasingly recognized for their dynamic role in gene regulation, which is mediated by covalent modifications to the histone tail domains as well as the incorporation of histone variants. The most common modifications include the addition of methyl or acetyl groups to lysine residues, although additional modifications such as phosphorylation, sumoylation, and ubiquitination are also important (Kouzarides 2007). Each modification on a specific amino acid is predicted to have its own unique outcome on gene expression; however, the combination of multiple modifications on the same or different nucleosomes is believed to dictate the transcriptional consequences. Certain modifications promote chromatin compaction, thereby blocking accessibility of the DNA to the transcriptional machinery, while other modifications

promote open chromatin conformation, which is permissive for gene activation. Furthermore, histone modifications also differ in their genomic context, as certain marks are prominent in the regulation of promoters, while others are important in regulating enhancers. The key histone modifications and their respective regulatory functions are outlined in Table 1.1.

The chromatin landscape of human islets

With the growing recognition that chromatin signatures are predictive of cell-type specific regulatory elements, there have been several efforts to define the epigenomic landscape of human islets. The first wave characterized regions of open chromatin that are likely to harbor active regulatory elements using high-throughput sequencing following formaldehyde-assisted isolation of regulatory elements (FAIRE-seq) (Gaulton et al. 2010) and mapping of regions sensitive to DNAseI (DNAse-seq) (Stitzel et al. 2010). These methods independently established that at least 40% of regions identified as open chromatin were specific to human islets. Unexpectedly, most of these islet-specific open chromatin domains did not overlap with known transcription start sites, but rather mapped to distal intergenic regions. The identification of putative islet regulatory elements was further refined by the integrative analysis of open chromatin regions, histone modification marks, transcription factor binding sites, and mRNA expression data for human islets and non-islet cells (Pasquali et al. 2014). This integrative approach allowed for the classification of various putative regulatory elements in human islets, including active promoters, active enhancers, and inactive or poised enhancers. These different studies conclusively demonstrate that distal enhancers, rather than promoters, are the likely drivers of tissue-specific gene expression in human islets. A striking observation made by all these groups is that these newly defined distal regulatory elements are enriched for SNPs associated with T1DM and T2DM disease risk (Gaulton et al. 2010; Stitzel et al. 2010; Pasquali et al. 2014), suggesting that these genomic variations may affect enhancers that in turn, may impact the activity of one or more nearby genes.

Due to the technical limitations of isolating human α- and β-cells, defining the cell-type specific chromatin signature of these cells has been more challenging. Nevertheless, using a panel of cell-surface antibodies to isolate highly purified human α- and β-cells (Dorrell et al. 2008), Bramswig *et al* determined the chromatin and transcriptional profile of these individual cell types (Bramswig et al. 2013). This study identified thousands of genes that were bivalently occupied by both activating and repressive histone modification marks in one cell type, but were monovalently marked in the other. This chromatin signature suggests that cellular reprogramming may be achieved through epigenetic manipulation of histone modifications at specific genes. Thus, to fully appreciate the regulation of gene expression in human islets and its relevance to disease, it will be necessary to characterize these newly recognized regulatory elements and their potential long-range interactions in α- and β- cells.

DNA Methylation

DNA methylation is a stable, yet reversible modification on the fifth position of cytosines, that mostly occurs in CG dinucleotides. This epigenetic mark is predominantly associated with gene repression (Siegfried et al. 1999), and is established and maintained by the conserved DNA methyltransferase enzymes (DNMT), DNMT1, 3A and 3B. Although the vast majority of CpGs in the mammalian genome are methylated, a small fraction occurs in CG-dense regions called CpG-islands that are largely resistant to DNA methylation. CpG islands typically overlap with promoters and regions of regulatory potential (Smith and Meissner 2013). In addition, many enhancer elements also have low levels of DNA methylation and are regulated in a cell-type specific manner (Sheaffer et al. 2014; Avrahami et al. 2015).

Imprinting refers to the biased expression of genes depending on the parental origin of the chromosome. This process is tightly regulated, typically through DNA methylation at *cis*-acting elements known as “Imprinting Control Regions” (ICR), to establish and maintain mono-allelic expression of specific genes (Thorvaldsen and Bartolomei 2007). Methylation at the ICRs is maintained despite active demethylation and dynamic reprogramming in the newly formed

zygote, and is only altered during establishment of novel methylation patterns in a sex-specific manner during primordial germ cell development (Bartolomei and Ferguson-Smith 2011). Imprinted loci are generally found in large clusters, where both maternally- and paternally-expressed genes are interspersed. Frequently, the protein-coding genes are expressed from one parental allele, while non-coding genes are expressed from the other (Barlow 2011).

While imprinting is most extensively studied in the context of fetal development, tissue-specific regulation in adult tissues has also been observed (J. T. Lee and Bartolomei 2013; Barlow 2011). As a result, several imprinted genes are also implicated in human diseases, where the imprinting defect arises in somatic tissues. One such example is that of the maternally-expressed adipose tissue transcription factor, *KLF14* (Parker-Katiraee et al. 2007), which is associated with risk for both T2DM and high-density lipoprotein disorders (Teslovich et al. 2010; Voight et al. 2010; Small et al. 2011). Perhaps the functionally haploid nature of these loci results in their increased likelihood to be associated with susceptibility to disease, as mutations in these genes, when found on the maternal chromosome that is expressed, cannot be "covered" by the gene from the other, silenced paternal allele. This may be particularly true for metabolic disorders, as several imprinted genes encode dosage-sensitive proteins related to growth factors and energy metabolism. Interestingly, several risk variants for T1DM and T2DM identified through GWAS are located in imprinted loci, including *KCNQ1*, *MEG3, PLAGL1* and *GRB10*. In fact, DNA methylation levels of the imprinted genes *MEG3* and *GNASAS* are altered in individuals periconceptually to famine, supporting the notion that imprinted regions are sensitive to environmentally mediated epigenetic alterations (Tobi et al. 2009).

<u>*DNA methylation in diabetes*</u>

DNA methylation has been demonstrated to be a critical regulator of β-cell identity, as a conditional deletion of *Dnmt1* in mouse β-cells results in transdifferentiation towards an α-cell fate due to derepression of the α-cell master regulator, *Arx* (Dhawan et al. 2011). Loss of *Dnmt3a* in β-cells results in their inability to appropriately secrete insulin in response to a glucose stimulus

(Dhawan et al. 2015). Additionally, the DNA methylation profile of β-cells has been found to change in an age-dependent manner, with methylation changes observed preferentially at enhancer elements (Avrahami et al. 2015). As the epigenetic mis-regulation of *Pdx1* has previously been associated with IUGR-related diabetes development, one group sought to evaluate *PDX1* methylation levels in islets from T2DM donors (Yang et al. 2012). Indeed, methylation levels at the distal promoter and enhancer were increased, correlating with decreased PDX1 expression levels. Similarly, aberrant DNA methylation has been associated with silencing of important genes, including the transcriptional coactivator *PPARGC1A,* and *INS* in T2DM donor islets (Ling et al. 2008; Yang et al. 2011). However, very few studies have comparatively profiled the entire methylome of non-T2DM and T2DM donor islets (Dayeh et al. 2014).

Overall, there is mounting evidence that establishment and maintenance of proper DNA methylation patterns is critical to β-cell function, although more thorough investigations of genome-wide methylation changes between islets from T2DM and control donors is required. The diagnostic value of dissecting cell type-specific methylation patterns is exemplified by the use of DNA methylation levels of a CpG site in the Insulin gene in circulating serum DNA as readout of β-cell destruction in T1DM (Akirav et al. 2011).

The *DLK1-MEG3* imprinted locus

The *DLK1-MEG3* imprinted locus on human chromosome 14 (mouse chromosome 12) embodies many features of a molecular candidate for diabetes etiology and heritability. This locus contains numerous miRNAs, lncRNAs, as well as protein-coding genes that have independently been recognized as abundantly expressed in rodent and human β-cells. Additionally, a SNP located in an intron of *MEG3* is associated with the risk for T1DM (Wallace et al. 2010). However, the regulation of this locus in β-cells and its potential involvement in diabetes pathogenesis has not been directly explored.

The maternally-expressed genes at this locus are all non-coding RNAs, consisting of the lncRNA, *Maternally Expressed Gene 3* (*MEG3*, known as *Gtl2* in mice), as well as a large cluster of microRNAs and snoRNAs (Schmidt et al. 2000; da Rocha et al. 2008; Seitz et al. 2004). In several tissues, the non-coding RNAs are derived from a single transcript that initiates from the *MEG3* promoter (Tierling et al. 2006; da Rocha et al. 2008). The paternally-expressed genes *DLK1, RTL1,* and *DIO3* all encode proteins. An antisense transcript of *RTL1* (*RTL1as*), expressed from the maternal allele, harbors miRNAs miR-127 and miR-136 that are maternally transcribed (Seitz et al. 2003). Due to their perfect complementarity to the *Rtl1* gene, it has been proposed that these miRNAs may function to silence *Rtl1* by RNA interference (Seitz et al. 2003). *DLK1* and *MEG3* are expressed in human fetal α- and β-cells, however their expression is restricted to β-cells in the adult islets at much higher levels than in the fetal samples (Blodgett et al. 2015).

Reciprocal imprinting is established by methylation of two differentially methylated regions (DMRs) on the paternal allele, one located ~13kb upstream of the *MEG3* transcription start site (Intergenic or IG-DMR), and the other overlapping with the promoter of the *MEG3* poly-cistronic transcript (*MEG3*-DMR) (figure 1.2). While the IG-DMR is the primary ICR for this imprinted cluster, the *MEG3*-DMR is also critical for regulating and maintaining imprinting at this region (Kagami et al. 2010). Failure to maintain imprinting at this locus can lead to either maternal or paternal uniparental disomy (UPD) of chromosome 14, which causes distinct and severe developmental disorders (Kagami et al. 2008). Several mouse models of *Meg3* deletion have been derived that recapitulate the severe developmental effects of loss-of-imprinting observed in humans. *Meg3* was first characterized as a non-coding RNA by a mouse in which a LacZ reporter was integrated 2kb upstream of this gene by insertional mutagenesis (*Gene trap locus 2*) (Schuster-Gossler et al. 1998). Insertion of exogenous sequences overlapping with the *Meg3*-DMR results in partial neonatal lethality, and surviving mice exhibit dwarfism when paternally inherited, although this paternal phenotype and related change in imprinted gene expression is dependent upon the mouse strain analyzed (Schuster-Gossler et al. 1998; Steshina et al. 2006). On the other hand, maternal inheritance of these transgenes results in a loss-of-imprinting

regardless of the background strain, but does not produce an obvious phenotype. Mice harboring a deletion of *Meg3* exons 1-5 exhibit varying phenotypes depending on the nature of the deletion and background strain of the mice, but in general exhibit peri- and post-natal death when the mutation is inherited from the mother (Takahashi et al. 2009; Yunli Zhou et al. 2010). These phenotypes underscore the importance of imprinting at the *Dlk1-Meg3* locus for normal embryonic development and survival.

Increased methylation of the *MEG3*-DMR and related loss of *MEG3* expression has been observed in several human cancers, such as pituitary and renal cell cancers and multiple myeloma (Zhao et al. 2005; Kawakami et al. 2006; Benetatos et al. 2008). These studies, coupled with *in vitro* experiments, suggest that *MEG3* functions as a tumor suppressor by activating p53, in a manner dependent upon the secondary structure of the *MEG3* RNA (Yunli Zhou et al. 2007; Yunli Zhou, Zhang, and Klibanski 2012). Furthermore, decreased expression of *MEG3* and the linked miRNAs with concomitant hyper-methylation of the DMRs may single-handedly explain the subtle phenotypic differences between induced pluripotent stem cells (iPSCs) and embryonic stem (ES) cells, such as the decreased efficiency in generating chimeric mice from iPSCs (Stadtfeld et al. 2010).

In a recent study, it was demonstrated that *Meg3* expression is decreased in mouse models of both T1DM and T2DM (You et al. 2015). Using siRNA-mediated transient repression of *Meg3 in vitro* and *in vivo*, the authors demonstrate that *Meg3* suppression results in decreased expression of the key islet transcription factors *Pdx1* and *MafA*, and consequently, of insulin. As a result, these islets exhibit impaired glucose-stimulated insulin secretion. Additionally, down-regulation of *Meg3* results in increased susceptibility of the immortalized Min6 β-cell line to apoptosis. Thus, this study established a critical role for *Meg3* in β-cell function and viability.

Embedded within the maternal transcript are at least 54 miRNAs (Seitz et al. 2004), making this one of the largest miRNA clusters in the mammalian genome. The islet and β-cell specific expression of miR-127, one of the miRNAs in this imprinted cluster, has been described by multiple studies. This miRNA had previously been identified as an islet-enriched miRNA in

rodent and human islets relative to acinar cells (Bravo-Egana et al. 2008), and as highly expressed in human fetal pancreata (Correa-Medina et al. 2009). Expression of miR-127 was also found to correlate with insulin expression levels and insulin secretion (Bolmeson et al. 2011). Finally, maternal deletion of the large miRNA cluster results in neonatal death and severe hypoglycemia in the surviving mice (Labialle et al. 2014). Thus, the miRNAs in the *DLK1-MEG3* locus have been implicated in glucose homeostasis; however, their targets have yet to be determined.

The protein-coding genes in this imprinted region include delta-like homolog 1 (DLK1), a non-canoncial Notch ligand that is expressed as a transmembrane protein whose extracellular domain is cleaved to yield a soluble factor known as fetal antigen 1 (FA1). *Dlk1* is widely expressed during development (Yevtodiyenko and Schmidt 2006), but is restricted to fewer tissues by adulthood (Falix et al. 2012). Due to this expression pattern and its known role as a negative regulator of Notch signaling, *Dlk1* is thought to be in important mediator of cellular maturation for many tissues (Falix et al. 2012; Appelbe et al. 2013). *Dlk1* is a well-established negative regulator of adipocyte differentiation (Smas and Sul 1993; Mitterberger et al. 2012; Abdallah, Beck-Nielsen, and Gaster 2013). Overexpression of the soluble form of *Dlk1* in many metabolically relevant tissues, including adipocytes, hepatocytes, and osteoblasts results in hyperglycemia and insulin resistance (K. Lee et al. 2003; Abdallah et al. 2015). Conversely, *Dlk1* null mice exhibit hyperinsulinism and increased β-cell mass (Abdallah et al. 2015). *DLK1* is highly expressed in human and mouse β-cells (Dorrell et al. 2011; Appelbe et al. 2013; Tornehave et al. 1996). *DLK1* was demonstrated to be stimulated by growth hormone and prolactin expression in proliferating rat islets, including during pregnancy; however, it is not directly responsible for the mitogenic effects of these hormones on islets (Carlsson et al. 1997; Friedrichsen et al. 2003). Of note, loss of expression of *Dlk1* in unchallenged mouse β-cells did not cause any observable phenotype (Appelbe et al. 2013) although these mice were not characterized for postnatal metabolic defects. It is plausible that the soluble and membrane-bound forms of *Dlk1* have different effects on glucose homeostasis (Falix et al. 2012).

The paternally-expressed gene *Rtl1* (*Retrotransposon-like 1*) is critical for normal placental development, and its loss results in severe developmental defects and neonatal lethality (Sekita et al. 2008). Similarly, Type 3 deiodinase (*Dio3*) is necessary for proper thyroid hormone regulation, and lack of *Dio3* results in partial perinatal lethality and abnormal thyroid hormone clearance in surviving mice (Hernandez et al. 2006). Consistent with the expression of the other genes in this imprinted locus, *Dio3* is expressed in the developing fetal pancreas and in mature mouse and human β-cells (Medina et al. 2011). Interestingly, mice lacking *Dio3* exhibit a mild decrease in β-cell mass, and become glucose intolerant by adulthood due to impaired insulin secretion, suggesting an important role for *Dio3* in β-cell function (Medina et al. 2011).

Overall, there is compelling evidence that genes in the *DLK1-MEG3* locus are important regulators of metabolism and glucose homeostasis. However, these genes have not been directly assayed for their role in human diabetes pathogenesis. Moreover, despite the high expression of this locus in β-cells, little is known about the mechanism by which mono-allelic expression is established. In this study, we analyze the differential expression of miRNAs between T2DM and control human islets, and identify the *DLK1-MEG3* genes as important molecular candidates for the pathogenesis of T2DM.

Tables

Table 1.1

HISTONE MODIFICATION	PREDICTED REGULATORY EFFECT
H2A.Z	Histone variant associated with dynamic chromatin, typically at regulatory elements
H3K4me1	Associated with regulatory elements, predominantly enhancers
H3K4me2	Associated with regulatory elements, such as promoters and enhancer
H3K4me3	Associated with active promoters and transcription start sites
H3K9me3	Repressive marks associated with heterochromatin
H3K27ac	Marks active, rather than poised or silenced, regulatory elements
H3K27me3	Repressive mark associated with transcriptional silencing
H3K36me3	Elongation mark associated with transcribed genes

***Table 1.1**:* Summary of a few important histone modification marks or variants and their proposed function in gene regulation. Modified from (ENCODE Project Consortium 2012).

Figures

Figure 1.1

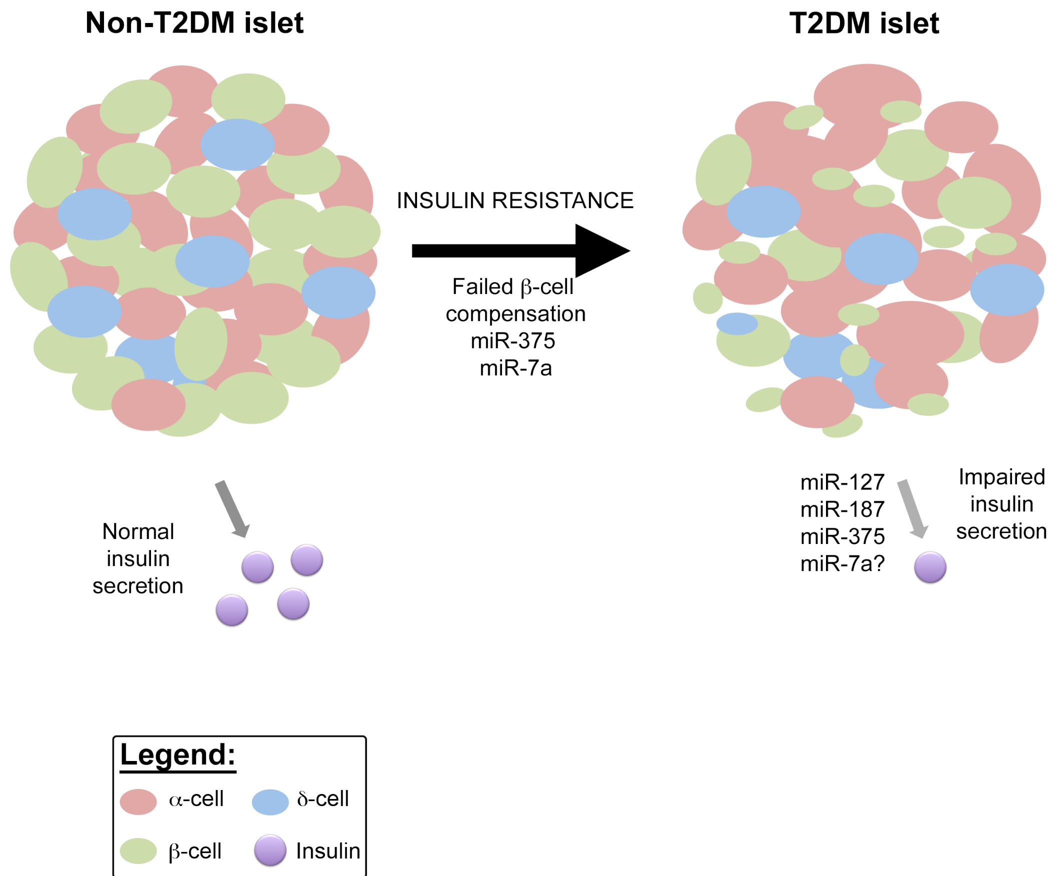

Figure 1.1: Schematic representation of the proposed role of the discussed miRNAs in glucose homeostasis.

Figure 1.2

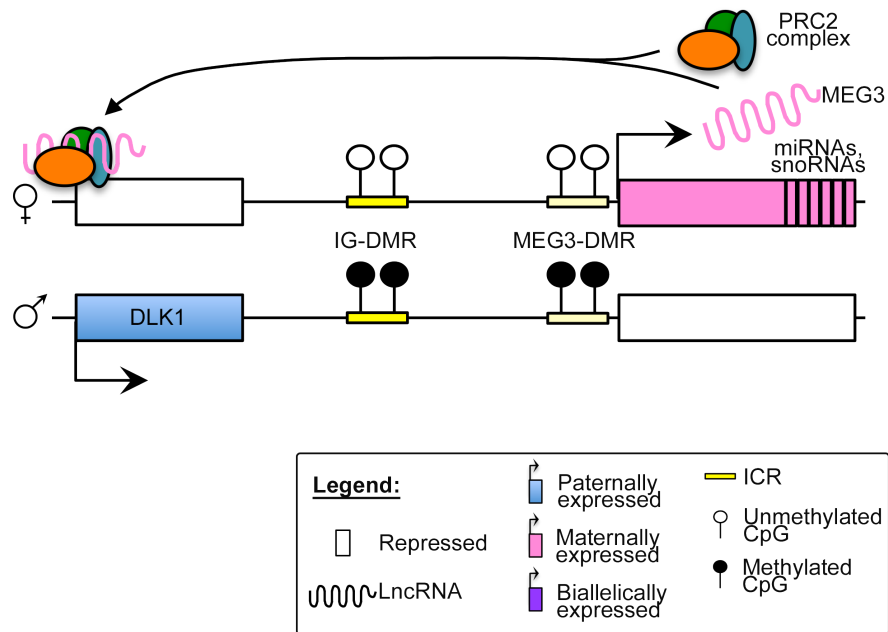

***Figure 1.2**:* Proposed model of imprinting at the *DLK1-MEG3* locus: The *DLK1-MEG3* imprinted region contains a primary (IG-DMR) and secondary (*MEG3*-DMR) ICR that overlaps with the promoter of *MEG3*. Both ICRs are paternally methylated. In mouse ES cells, the *Meg3* lncRNA is believed to direct PRC2 mediated silencing of *Dlk1* (Zhao et al. 2010)

Chapter 2

Materials and Methods

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. Total and small RNA was isolated from the islets by using the mirVana miRNA Isolation Kit (Ambion Cat# AM1560).

miRNA sequencing & comparison

The isolated microRNA from seven samples (three non-diabetic donors and four type 2 diabetic donors) 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).

After preparation and sequencing of amplified libraries, we trimmed 3' Illumina adapter sequences from the sequence read. The resulting unique sequences with lengths between 15-36 base pairs were aligned to the human genome, RefSeq and to mirBase (release 20.0) using Illumina's ELAND program to determine the content of our samples. Insertions/deletions and mismatches were excluded and the counts for all reads with lengths between 19 and 25 (inclusive) were summed to obtain the expression of known miRNAs in each sample. When a trimmed read matched perfectly to two or more mature miRNA forms these forms were grouped together in a single ad hoc family.

Differential analysis consisted of two major components: normalization and statistical evaluation of differences between the groups. Relative read counts (in reads per million) for the expression level of the miRNA (family) genes in each replicate were normalized using quantile normalization using a limma package (normalizeBetweenArrays (Gentleman et al. 2004; Smyth and Speed 2003)), The resulting normalized intensities were then passed to SAM (Significance Analysis of Microarrays (Tusher, Tibshirani, and Chu 2001)) to determine an FDR significance

value. We used a false discovery rate of 20% and a minimum fold change of 1.5x 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 50 bp on an Illumina hiSeq2000.

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. 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 Table 2.1) 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. 2008).

HITS-CLIP 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 (UCSC hg19 assembly) using a previously described analysis pipeline (F. Li et al. 2012). 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.

The adapter trimming parameters were set to ≤15% mismatch of ≥8nt alignment to the 3' of the reads; reads mapping parameters were carefully tuned to allow ≤10% or ≤8% total mismatches for miRNA and mRNA library, respectively; only those alignments with ≤4% mismatches compared to the best hits were retained. As a supplement, we also mapped the

mRNA reads to RefSeq mRNA sequences (Jul, 2012 release) to recover exon-exon junction mapped reads using the same parameters as above.

To illustrate the global miRNA target preference between CDS and both UTR regions, we calculated the read coverage for each nucleotide of every human islet transcript using all mapped mRNA library reads; Islet transcript was defined as with at least one mapped read from the target library. Then we equally divided islet transcripts into 100 “bins” for 5’UTR, CDS and 3’UTR separately. The average read depth of all nucleotides falling into each bin was used as the “average read coverage”, and averaged again across all islet transcripts.

Identification of Argonaute footprints and their interaction with miRNAs

To identify Argonaute footprints, we first assembled all mapped mRNA reads to consecutive genomic contigs as a starting point. To exclude low-abundance contigs that might come from non-specific binding to the Argonaute protein, we then characterized highly expressed contigs as final Argonaute footprints using a Poisson distribution based statistical model. To be specific, we first calculated the normalized RPKM expression values for every contig, then fitted them to the Poission distribution as

$$f(x;\lambda) = \Pr(RPKM = x) = \frac{\lambda^x e^{-\lambda}}{x!},$$

where the parameter λ was estimated using the mean RPKM value of all contigs. Contigs with significant high RPKM values ($p<0.05$) were defined as Argonaute footprints. As a result, we identified a total of 12,492 Argonaute footprints with an average length of 98.3 nt in human islets.

Chimeric reads analysis

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 (Morán et al. 2012)).

Dual luciferase reporter assay – miRNA targeting assays

$6x10^4$ 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 tough decoys

Tough decoys were synthesized as described in (Haraguchi, Ozaki, and Iba 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 $5x10^7$-$1x10^9$ TU/ml. Groups of 200-250 islets were transduced overnight with $5x10^3$ TU/islet with 4μg/ml of polybrene. Media was replaced every day and RNA was extracted 72 hours post transduction for RT-qPCR.

Dual luciferase reporter assay – methylation sensitivity assay

The promoters of human *MEG3* (hg38 - chr14:100,824,286-100,826,471) and CDKN2A (chr9:21,974,864-21,975,618) were cloned into the pCpGfree-Lucia vector (Invivogen). 5μg of empty vector or the promoter constructs were methylated *in vitro* using M.SssI CpG methylase (Zymo Research) according to manufacturer's instructions. Complete *in vitro* methylation was verified using HpaII digestion of 500ng plasmid DNA. The luciferase constructs (methylated and unmethylated) were transfected into βTC6 cells seeded at 125,000 cells/well of a 24-well plate along with 25ng of pRL-SV40 (Promega) using FuGENE HD transfection reagent (Promega). Cells were harvested 48 hours post-transfection and processed for luciferase readout as described above. Experiments were performed three times with three technical replicate wells per experiment. The experiment was also performed in HEK293T and HeLa cells using 75,000 cells/well and by harvesting the cells 24 hours post-transfection.

TALE experiments

TALEs targeting the mouse *Meg3*-DMR (chr12:109,540,635-109,540,653) were designed using an online resource and as described before (D. L. Bernstein, Le Lay, et al. 2015). $12x10^6$ βTC6 cells in a 10cm dish were transfected with 12μg of either TALE WT or mutant plasmids using FuGENE HD transfection reagent (Promega). Cells were FACS-sorted for GFP+ cells after 72 hours on Diva 206 (Penn Flow Cytometry and Cell Sorting Facility). Total RNA and genomic DNA

were extracted from the collected cells using an Allprep DNA/RNA mini kit (Qiagen). DNA methylation analysis was conducted as described above. The primers used to quantify DNA methylation levels by pyrosequencing are listed in Table 2.2.

Dual luciferase reporter assay – enhancer activity assay

The human *MEG3*-DMR (hg38 - chr14: 100,824,307-100,826,452) and *MEG3* enhancer (chr14:100,842,082-100,843,068) were subcloned into pGL3-Basic or pGL4.23[*luc2*/minP] luciferase reporter (Promega). 50,000 βTC6 cells were seeded per well of a 24-well plate and transfected with 500ng of plasmid DNA and 10ng of pRL-SV40 (Promega). Cells were harvested 24 hours post-transfection and processed for luciferase readout as described above. Experiments were performed in triplicates with four technical replicates per experiment.

Allele-specific ChIP-PCR

Allele-specific ChIP was performed according to the schema in figure 4.11A. 30ng of genomic DNA from islet donors was used to identify donors heterozygous for the SNP rs3783355. SNP genotyping was performed using Taqman SNP genotyping Assay (ThermoFisher Scientific C_1259770_10, Cat #4351379) and Taqman genotyping Master Mix (ThermoFisher Scientific Cat# 4371353) on a Stratagene Mx3000P thermocycler. Chromatin was extracted from non-diabetic donors' islets as previously described (Bramswig et al. 2013). The ChIP antibodies and conditions used for this experiment were described by (Pasquali et al. 2014). The primers used for this experiment are listed in Table 2.3. Following PCR of the input and ChIP DNA, libraries were prepared using the NuGEN Mondrian SP+ system and sequenced on an Illumina MiSeq to obtain approximately 200,000 reads per library (sequence read count ranged from 24,568 to 916096 reads). For the qualitative assay, 300ng of PCR amplified input or FOXA2 ChIP DNA was digested using the restriction enzyme BanII. Uncut PCR product (150 bp) and BanII digested PCR products (113 bp + 37 bp fragments) were run on a 3% gel to visualize differences.

Circular chromosome conformation capture (4C-Seq)

4C-Seq was performed on ~10,000 IEQ of human islets using the enzymes DpnII (first enzyme) and NlaIII (second enzyme) according to (van de Werken et al. 2012). The libraries were prepared using the BiS-PCR^2 protocol (D. L. Bernstein, Kameswaran, et al. 2015). Libraries were sequenced on an Illumina HiSeq on Rapid Run Mode to obtain 100 bp sequences (single-end reads). Five pooled libraries were sequenced per lane, with 20% PhiX supplemented to increase read diversity. Primers used to determine DpnII digestion efficiency and for library preparation are listed in Table 2.4.

Accession numbers

All miRNA sequencing datasets have been deposited into NCBI GEO under accession number GSE52314. All HITS-CLIP library sequencing data have been deposited into NCBI GEO under accession number GSE51924.

Tables

Table 2.1

Region	Assay name		Primer sequence
1	CG4_Assay1	Forward	5'Biotin-ATTATTGAATTGGGTTTGTTAGTAGT
		Reverse	ATCAAAACAACTCAAATCCTTTATAAC
		Sequencing	CCTTTATAACAAATTAAAATATATC
2	CG4_Assay2	Forward	GTTTTATTATTGAATTGGGTTTGTTAGTA
		Reverse	5'Biotin-ATCAAAACAACTCAAATCCTTTATAAC
		Sequencing	AATTGGGTTTGTTAGTAG
3	CG7_Assay1	Forward	5'Biotin-ATTATAGGGTGTTGGTTATGG
		Reverse	CCCCAAATTCTATAACAAATTACTCT
		Sequencing	CAACAAAAAAAAAAAAAAAAAAATTC
4	CG7_Assay2	Forward	5'Biotin-TTAGATTGTAGTAAAGAAGGGAGGAAAAAA
		Reverse	CCCCCACACATTATACCTAAATTC
		Sequencing	ATTATACCTAAATTCACCCT

***Table 2.1**:* Sequences of primers used in pyrosequencing assay for the human IG-DMR and *MEG3*-DMR

Table 2.2

Assay name	Region		Primer sequence
*Meg3*_DMR	1	Forward	GTATAGAAGAAGAAGAGTTGGAATAGAGTT
		Reverse	5’ Biotin-TAAAAAAAAATCCCCAACACTAACCTT
		Sequencing	GAAAGGATGTGTAAAAATGA
*Meg3*_DMR	5	Forward	GGTTTTGGTGGTTGAAAGTT
		Reverse	5'Biotin- AATAACTCCAAACCCCCCTTTTCAAA
		Sequencing	GTGGTTGAAAGTTTTTTTTAGA
*Meg3*_DMR	4	Forward	AGAAAAGGGGGTGTGTGGA
		Reverse	5’ Biotin-AAACTACCCCCCCCCTCAA
		Sequencing	GTAGGGTTTTTTTTGGTATT
IG-DMR		Forward	5'Biotin-TTAGATTGTAGTAAAGAAGGGAGGAAAAAA
		Reverse	CCCCCACACATTATACCTAAATTC
		Sequencing	ATTATACCTAAATTCACCCT
Dlk1	1	Forward	TTGGTTTGGTTTTTGAGATTT
		Reverse	5’biotin-AATCCCCATAACCCACCCCCTAA
		Sequencing	GTAGGTATTAGTTTAGTTAAAGAGT
Dlk1	2	Forward	GGTGTAATTTTGGTTTTTTTTT
		Reverse	5’biotin-AAAACTCACCATAAATACTATAACC
		Sequencing	GTAGTTTTAGGAGTAGTT
CpG 28		Forward	AAGGTGTTAATAGGTGGATTTTATA
		Reverse	5’biotin-ATAACCTCTCTCTAACCCCTAAAAA
		Sequencing	GTTAATAGGTGGATTTTATATAAGT
CpG 98	1	Forward	AAGTTTAGTTTATTGGAGTATTTGT
		Reverse	5’ Biotin-AAAAATCCTTTCATATCCTTAACCT
		Sequencing	AGGGTAAAGAAGGTG

CpG 98	2	Forward	AGTGGGGGAATTTAGTTATTTAAG
		Reverse	5’ Biotin-ACCACACTAATAAACCCT
		Sequencing	ATTGTGTTGTGGATAGTTAAA
Dio3	1	Forward	GTTGTTGGGGAGTTGTTT
		Reverse	5’ Biotin-CTCCCACTAAAAATAACTATTACCT
		Sequencing	GTTTTAGGGGTAGAGAGTAA
Dio3	2	Forward	GGGGAATTTAGTTTAGGT
		Reverse	5’ Biotin-ACCAATACCCCAAAAATCTTA
		Sequencing	GGGAATTTAGTTTAGGTG

Table 2.2: List of pyrosequencing primers used to assay methylation levels across the *Dlk1-Meg3* *locus.*

Table 2.3

Amplified Site	Forward primer	Reverse primer	Control for ChIP
RS3783355	CGAGATCTGAGGCAAGACTG	GCACCTAACTGATCTGGCATG	
SLC30A8 PROM	CCGAAGGTCAAAACCCTACA	AGGGAGACTTGCAGGTAGCA	MAFB
SLC30A8 ENH	AGTGCTTCTGGAGGTGAGGA	TAGGATGTCAGAGGCCCATC	NKX6.1
GLIS3 INSP	CGGAAATTGCAGCCTCAG	CCCCGCTGGCTTTATAGTCT	PDX1
MAFB ENH	GCTCTGCCACCTAGGTTTGT	GTTGCTTAACGATGGGGAAA	NKX2.2
NR0B2 PROM	GCTGCCCCTTATCAGATGAC	CTGGCTTAGCAAAAGCCCTA	FOXA2

***Table 2.3*:** List of primers used for Transcription factor ChIP. The primers used as positive controls for ChIP were obtained from (Pasquali et al. 2014).

Table 2.4

Description	Sequence	Use
MEG3_DpnII_F1	GCTTGCATTTGTCAGGGGAA	DpnII digest check
MEG3_DpnII_R1	TAGAGCCCGTCGTTACACAA	DpnII digest check
MEG3_DpnII_F3	TTGTGTAACGACGGGCTCTA	DpnII digest check
MEG3_DpnII_R3	AGCACCTAACTGATCTGGCA	DpnII digest check
hsa_MEG3_enhF1	TTAGACGGGCAGACTGCTTT	DpnII digest internal control
hsa_MEG3_enhR1	CCTCTTTGCTTCAGGAGTGG	DpnII digest internal control
Enhancer read primer 2 + BiS PCR^2 adapter	ACACTCTTTCCCTACACGACGCTCTTCCGATCT GGCAAACAGCACCTAACTGATC	Primers for library prep
Enhancer reverse primer 2 + BiS PCR^2 adapter	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT GGAGTGTTCACCAATGCTTTCT	Primers for library prep
CpG45 read primer 1+ BiS PCR^2 adapter	ACACTCTTTCCCTACACGACGCTCTTCCGATCT TGTCGGGGCGCAGATC	Primers for library prep
CpG45 reverse primer 1+ BiS PCR^2 adapter	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT TTGTGCCTGAATTCACCCTG	Primers for library prep

Table 2.4: Primers used for human islet 4C-Seq. Red and green sequences indicate the overhangs added for library preparation based on BiS-PCR^2 (D. L. Bernstein, Kameswaran, et al. 2015)

Chapter 3

Epigenetic Regulation of the *DLK1-MEG3* MicroRNA Cluster in Human Type 2 Diabetic Islets

Abstract

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.

Parts of this chapter were adapted from Kameswaran et al. 2014. Epigenetic Regulation of the DLK1-MEG3 microRNA Cluster in Human Type 2 Diabetic Islets. Cell Metabolism 19 (1): 135–45.

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. According to the International Diabetes Federation, approximately 387 million people worldwide have T2DM. Several studies have indicated that T2DM has a high rate of familial aggregation (Drong, Lindgren, and Mccarthy 2012; Nolan, Damm, and Prentki 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, Lindgren, and Mccarthy 2012).

MicroRNAs (miRNAs), short non-coding RNAs that post-transcriptionally regulate gene expression, have emerged as strong molecular candidates in several complex diseases, in part due to their ability to simultaneously regulate the expression of hundreds of target mRNAs (Mendell and Olson 2012a). 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 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 donor 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 donor islets. Using high-throughput sequencing of cross-linked and immunoprecipitated RNA (HITS-CLIP) we identified targets of Chr 14q32 miRNAs, such as *IAPP*

and *TP53INP1*, with known association with the pathogenesis of T2DM. Additionally, we discovered 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*. Overall, our results provide strong evidence for a role of miRNAs and epigenetic modifications, such as DNA methylation, in the pathogenesis of T2DM. In addition, this data set catalogs human islet microRNAs relevant to human T2DM pathogenesis and elucidates their target transcriptomes.

Results

Differentially expressed miRNAs in T2DM human islets

To determine the miRNA transcriptome of the mature human endocrine pancreas, we isolated the small RNA fraction from islets of three non-diabetic and four T2DM organ donors (donor information available in table 3.1). 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. 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 figure 3.1A, 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 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 figure 3.1B and C. Hsa-miR-375, which was shown to be highly expressed in developing and mature human islets(Bolmeson et al. 2011; Mugdha V Joglekar and Hardikar 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, important regulators of insulin sensitivity in the livers of obese mice 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.

Comparative analysis of samples from T2DM donors and non-diabetic donors identified 15 miRNAs with significantly differential expression (figure 3.2A). 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 figure 3.2B. The differential expression was validated for 9 of these miRNAs by Taqman qRT-PCR on islets from 16 T2DM and 18 non-diabetic donors (figure 3.2C and D). MiRNAs miR-204 and -184, which have previously been

identified as 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, ruling out the possibility that the observed differential expressions are driven by changed islet composition between the two groups. Expression of the differential miRNAs identified in this study did not show a significant correlation with age, sex or body mass index (BMI) (figure 3.3).

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 negatively affect 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 donor 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 *DLK1-MEG3* 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 protein-coding 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 figure 3.6C below), we asked whether other miRNAs in this cluster were also expressed at lower levels in

T2DM donor islets. As shown in figure 3.4A, this was indeed the case, and these results were confirmed by Taqman qRT-PCR in a larger cohort of islet samples (figure 3.4B). *MEG3* was also 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.

<u>The DLK1-MEG3 cluster of miRNAs is specifically expressed in human β-cells</u>

To characterize the expression profile of the *DLK1-MEG*3 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 (figure 3.5). The expression levels of the miRNAs in the 14q32 cluster were, on average, 16-fold higher in β-cells than in α-cells (figure 3.6A and B). This is consistent with previous reports that utilized different sorting strategies and identified some members of the Chr 14q32 cluster of miRNAs to be enriched in human β-cells compared to α-cells (Klein et al. 2013) or whole islets (van de Bunt et al. 2013). Expression of the long non-coding RNA *MEG3* was also found to be 20-fold higher in β-cells than in 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 analyzed previously published ChIP-Seq data 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 (figure 3.6C). 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, our data demonstrates that 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-MEG*3 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 idea, 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 (figure 3.7), 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-MEG*3 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 the "*MEG3*-DMR") (figure 3.8). 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, representing the fully methylated paternal and unmethylated maternal alleles, we observed a decreased intensity of the unmethylated product in islets from donors with T2DM (figure 3.9A).

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 (figure 3.9B, 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 (figure 3.9C, 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 (figure 3.9D). 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 (figure 3.10A). From these deep-sequencing libraries, we identified 12,492 and 456 Argonaute-associated mRNA footprints and mature human miRNAs respectively. Among the mRNAs targeted by the RISC complex in human islets were several encoded by genes essential for islet function. 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) (Student's t-test, p-value = 7.00E-16) and 3'UTR (Student's t-test p-value = 5.26E-17), similar to what has previously been described for other tissues (Chi et al. 2009; Forman, Legesse-Miller, and Coller 2008; McKenna et al. 2010) (figure 3.10B and C).

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 found in the target library. 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 prior 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.

Since the expression of the *MEG3*-miRNA locus is down-regulated in islets of T2DM donors, we were particularly interested in targets detrimental to islet function when highly expressed. 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, Janson, Soeller, et al. 2003; Höppener 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 (figure 3.10D). 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 reference sequences, we discovered a unique class of sequences, termed chimeric reads, that partially mapped to both miRNAs and target mRNAs simultaneously. 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 (figure 3.10A). 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 very 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 figure 3.11A 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 figure 3.11B.

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” (figure 3.11D), 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 (figure 3.11E 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* (figure 3.12A). *TP53INP1* is the nearest gene to a T2DM risk-associated single nucleotide polymorphism in individuals of Caucasian descent (Voight et al. 2010). *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 (Yuedan Zhou et al. 2012). As expected, we observed an increase in *TP53INP1* mRNA levels when comparing T2DM to non-diabetic donor islets when assayed by both microarray (data not shown) and qRT-PCR, although these data did not reach statistical significance (figure 3.12B). 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, figure 3.12C). Next, we validated the miR-495 and *TP53INP1*

targeting relationship using luciferase reporter assays, as these assays provide a readout of miRNA effects at the mRNA and protein level (figure 3.12D). We observed a 20% decrease in luciferase activity in the presence of miR-495 mimic, but not the scrambled mimic. To further test this targeting relationship *in vivo*, we constructed 'tough decoy' RNAs (Haraguchi, Ozaki, and Iba 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 (figure 3.12E, $p = 0.007$), similar to the increase observed in T2DM donor islets (figure 3.12B). *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 multiple diseases (Mendell and Olson 2012b). To identify the miRNAs that might contribute to the pathogenesis of T2DM, we sequenced the small RNAs of islets obtained from healthy and T2DM organ donors. 15 miRNAs were significantly differentially expressed, with 5 miRNAs upregulated and 10 downregulated in islets of T2DM donors. Of particular interest was the maternally-expressed, imprinted cluster of non-coding RNAs on human chromosome 14q32, which was down-regulated in islets from T2DM donors. Our H3K4me3 and H3K27me3 histone modification mark data, 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 14q32 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. In fact, in a recent study, *Meg3* expression was found to be responsive to high glucose in mouse islets and in the Min6 β-cell line (You et al. 2015). This discrepancy may be a result of the different species and glucose concentrations used in the studies.

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. 2013; Voight et al. 2010).

We have integrated high-throughput sequencing of the human islet miRNA transcriptome 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.

Tables

Table 3.1

Donor ID	Age	Gender	Blood Type	BMI	Race	Cause of Death
Non T2DM 1	33	F	A+	31.1	AA	Anoxia, CVA
Non T2DM 2	22	F	O+	24.9	AA	Head trauma
Non T2DM 3	51	M	O+	26.1	Hispanic	Head trauma
T2DM 1	61	F	A-	29.6	Caucasian	Anoxia, CVA
T2DM 2	45	M	B+	37	Caucasian	Anoxia, CVA
T2DM 3	53	M	O+	38.4	Caucasian	CVA
T2DM 4	54	F	A	21.6	Hispanic	CVA

Table 3.1. *Islet donor information.* Characteristics of T2DM and non-T2DM islet donors used in small RNA-sequencing screen are listed. (Abbreviations: AA = African American, CVA = Cerebrovascular accident)

Figures

Figure 3.1

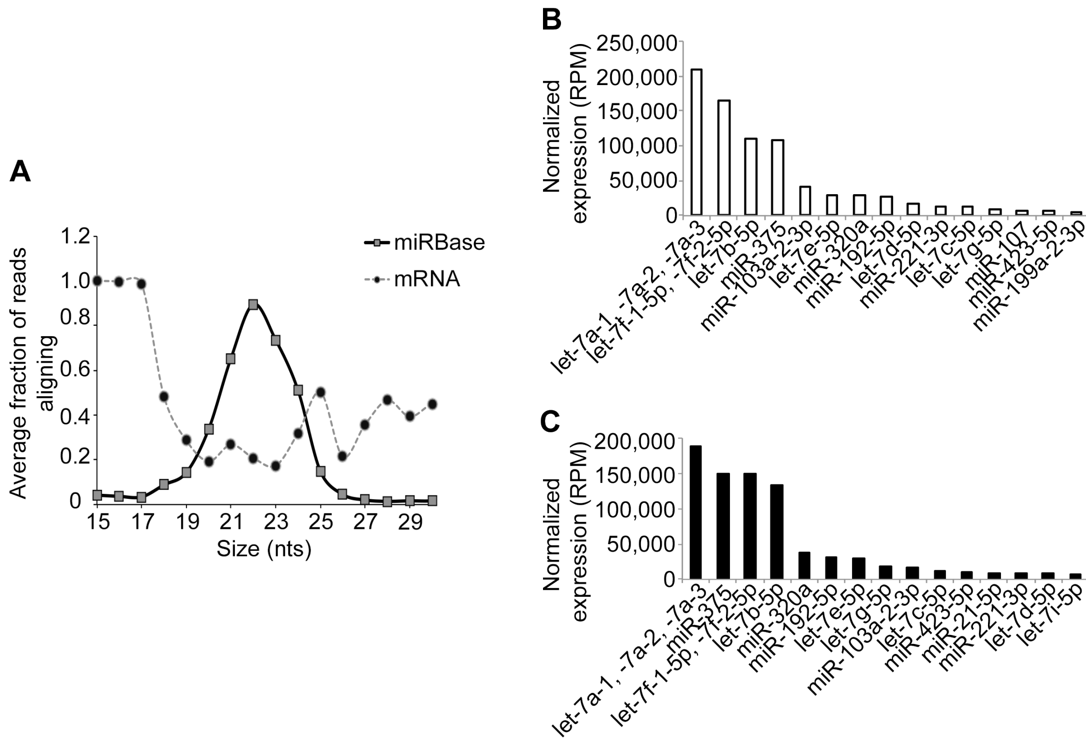

***Figure 3.1**: The miRNA transcriptome of human islets* (**A**) Small RNA sequencing reads alignment to miRBase and Refseq. Expression levels of the 15 most abundant miRNAs in (**B**) three non-diabetic and (**C**) four T2DM human islets as identified by small RNA sequencing.

Figure 3.2

Figure 3.2: *Differentially expressed miRNAs in T2DM human islets.* (**A**) Differentially expressed miRNAs between non-T2DM (n=3) and T2DM (n=4) islets, identified by a FDR of 20% and minimum fold change of 1.5x. MiRNAs up- and down-regulated in T2DM islets are highlighted. (**B**) Distribution of expression levels of miRNAs identified as significantly differentially expressed by small RNA sequencing across clustered samples. Taqman qPCR confirmation of (**C**) down-regulated and (**D**) up-regulated miRNAs in 16 T2DM and 18 non-T2DM human islets. *p*-value calculated using Student's T-test. *p<0.05, **p-value<0.01, ***p-value<0.005. Error bars indicate mean +/- SEM.

Figure 3.3

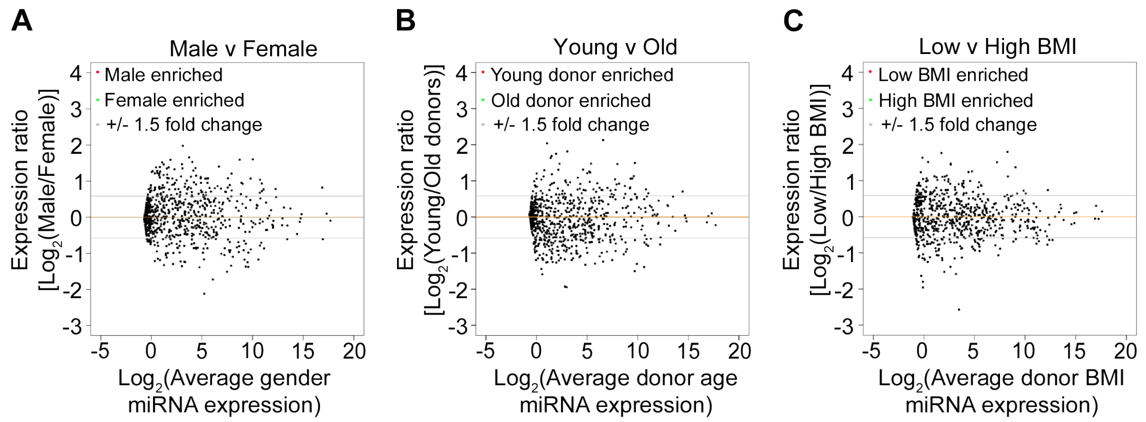

***Figure 3.3**: Differentially expressed miRNAs in T2DM do not correlate with other factors.* Human islet miRNA correlation with (**A**) Gender (**B**) Age (**C**) Body mass index (BMI). MiRNAs enriched or down-regulated in each category are highlighted in red and green respectively.

Figure 3.4

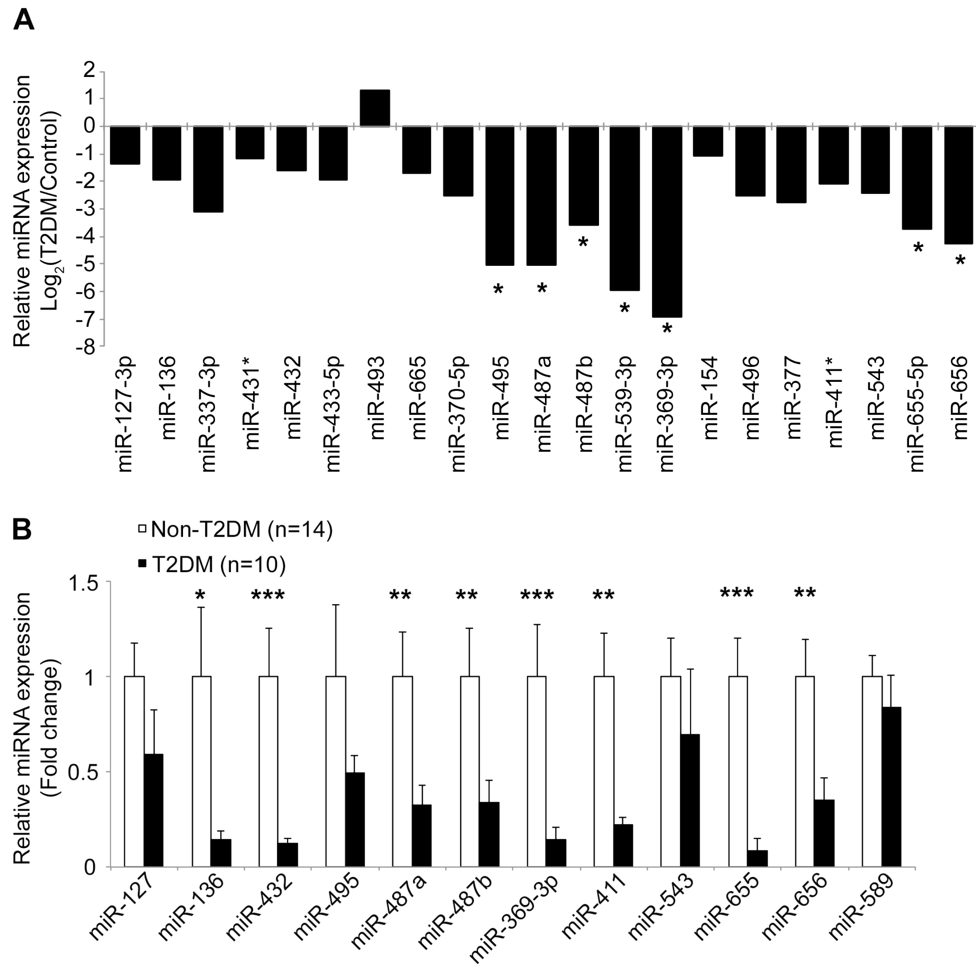

Figure 3.4: *The imprinted chromosome 14q32 miRNA cluster is down-regulated in T2DM islets* (**A**) Expression of Chr 14q32 miRNAs as determined by small RNA sequencing of T2DM (n=4) and non-T2DM (n=3) islets. (**B**) Relative expression of miRNAs in the Chr 14q32 cluster as determined by Taqman qPCR 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. Error bars indicate mean +/- SEM.

Figure 3.5

Figure 3.5: miRNAs that are differentially expressed between α- and β- cells as determined by small RNA sequencing. MiRNAs enriched in α-cells are highlighted in red, while miRNAs enriched in β-cells are marked in green.

Figure 3.6

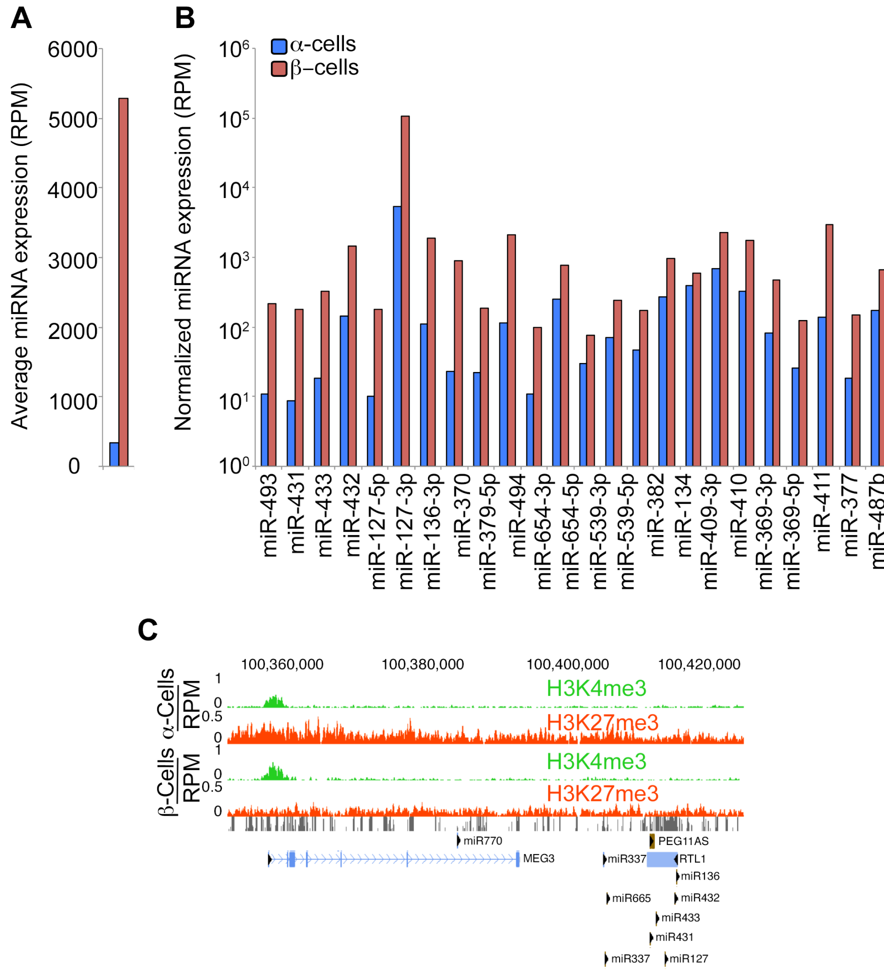

Figure 3.6: *Chr 14q32 miRNAs are highly and specifically expressed in human β-cells.* (**A**) Average expression of Chr 14q32 cluster miRNAs in human α- and β- cells. (**B**) Expression of Chr 14q32 miRNAs (minimal expression 50 reads per million (RPM)) in sorted human α- and β- cells. (**C**) Genome 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.

Figure 3.7

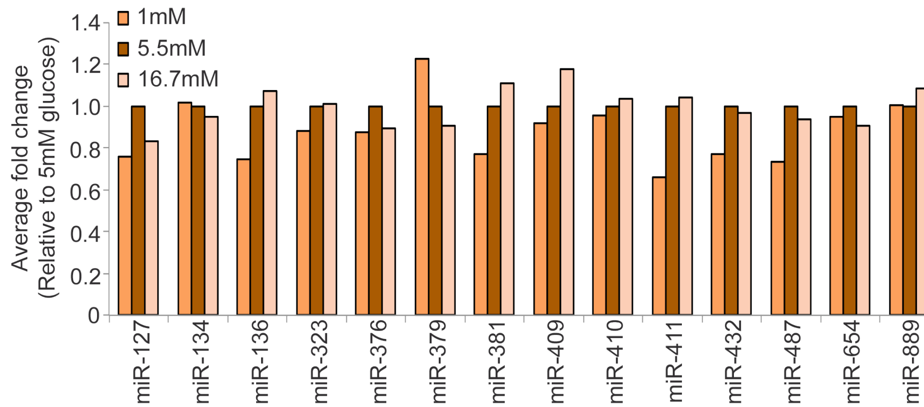

Figure 3.7: *Chr 14q32 miRNAs are not regulated by acute changes in glucose concentration.* Expression of *MEG3* miRNAs in non-diabetic islets (n=1) that were cultured in 1mM, 5.5mM and 16.7mM glucose for 16 hours. After small RNA sequencing, the expression values for each miRNA (for miRNAs >100rpm) was normalized to the 5.5mM value

Figure 3.8

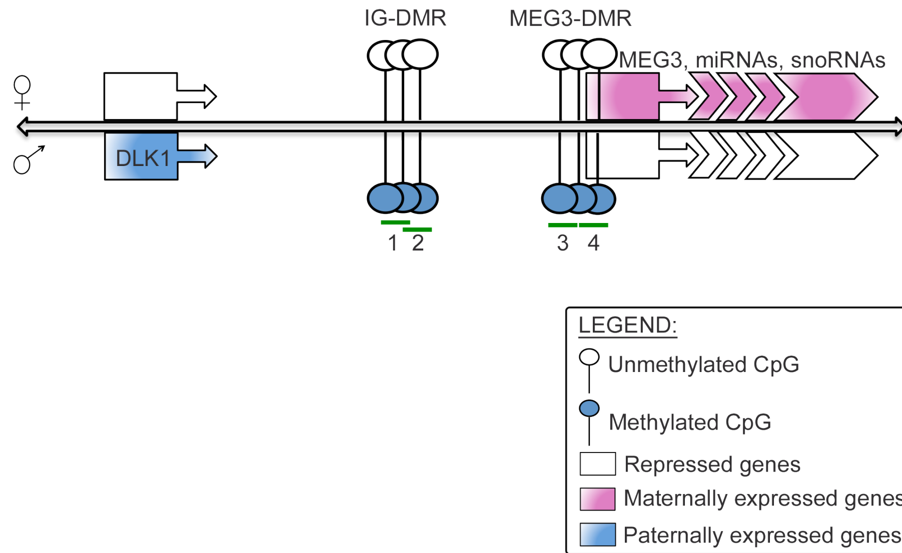

Figure 3.8: Schematic representation of *DLK1-MEG3* locus DMRs with allele-specific gene expression depicted. Regions analyzed for figure 3.9 B, C and D are marked as green bars.

Figure 3.9

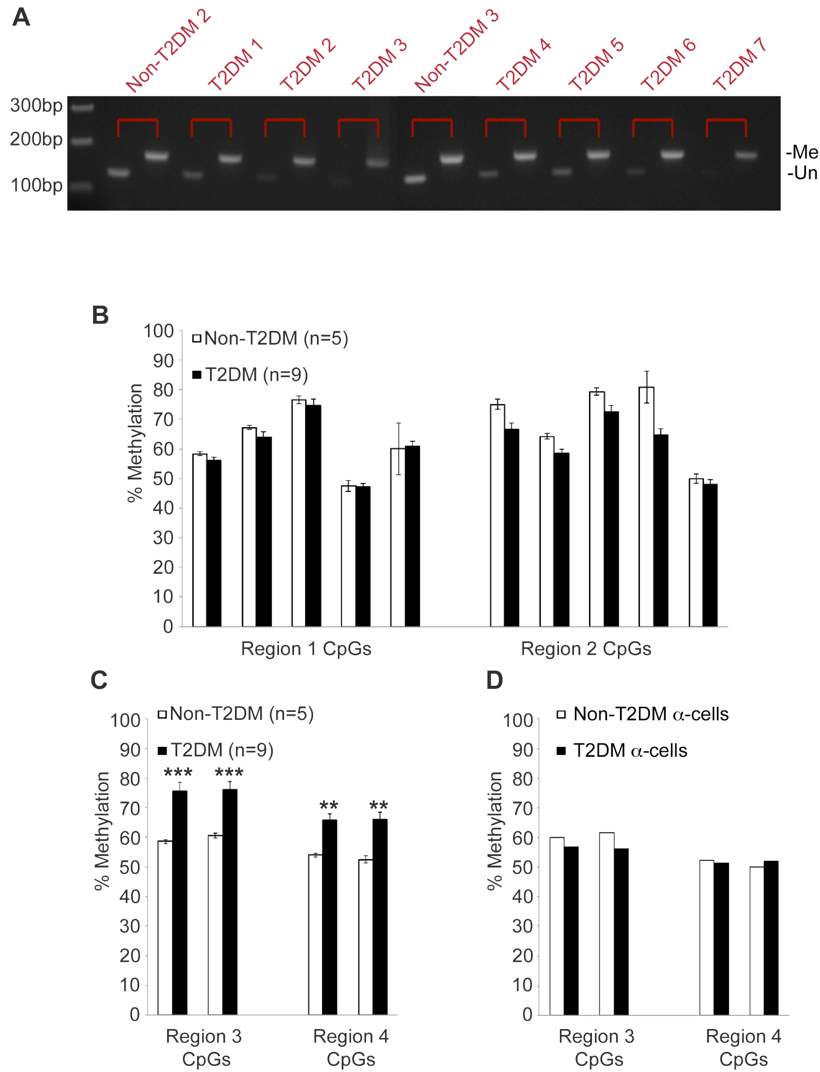

Figure 3.9: *Increased methylation of the MEG3-Differentially methylated region (DMR) in T2DM islets*. (**A**) Methylation specific PCR for the *MEG3* promoter on two Non-T2DM and 7 T2DM donors' islets. Unmethylated band (Un) is 120bp, Methylated band (Me) is 160 bp. Percent methylation was determined for multiple CpGs in the (**B**) IG-DMR and (**C**) *MEG3*-DMR in 5 non-diabetic and 9 T2DM donors' islets and (**D**) in the *MEG3*-DMR in α-cells sorted from a confirmed T2DM (n=1) and non-T2DM (n=1) donor by pyrosequencing of bisulfite converted DNA. Each bar represents an individual CpG, and the regions refer back to schema in figure 3.8. *p*-value calculated using Student's T-test. ***p*-value<0.005, ****p*-value<0.001. +/- SEM.

Figure 3.10

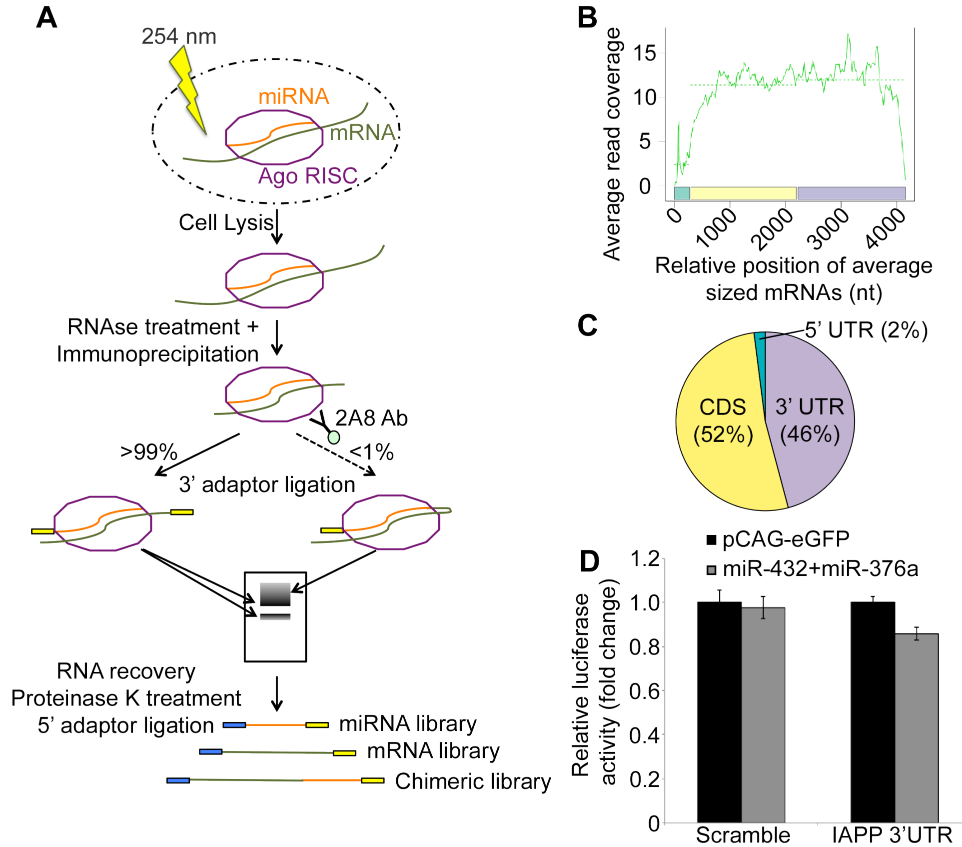

***Figure 3.10**: Identification of miRNA targets in human islets by HITS-CLIP.* (**A**) Schema of HITS-CLIP procedure and chimeric reads ligation. (**B**) Average read coverage of all HITS-CLIP target mRNA fragments over a standardized mRNA. (**C**) Argonaute footprint distribution across target library 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 x 10-5.

Figure 3.11

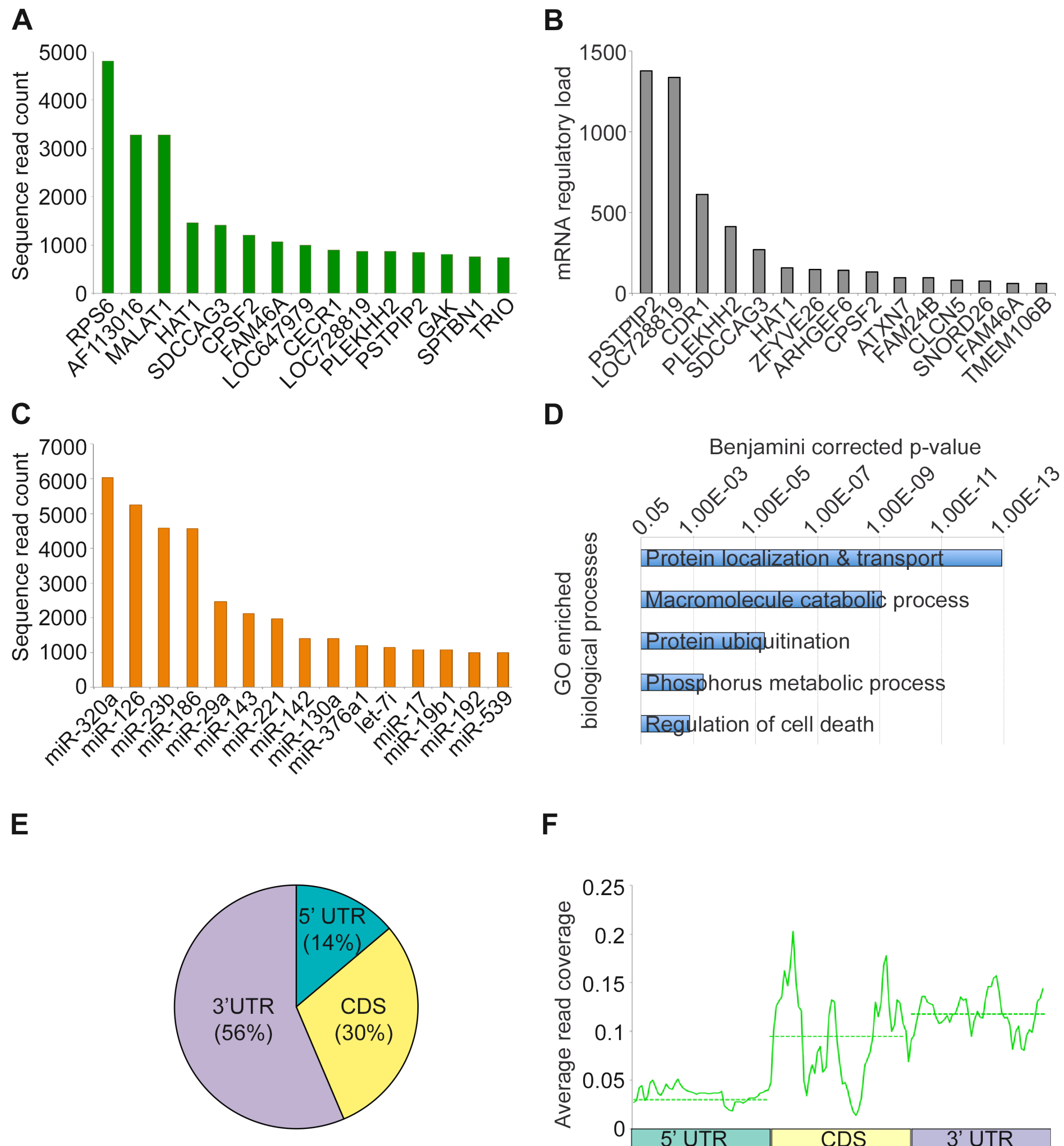

Figure 3.11: *Determination of direct miRNA:mRNA targeting relationship from chimeric reads.* Deep sequencing of our Argonaute HITS-CLIP library identified thousands of chimeric reads, consisting of a mature miRNA and a target mRNA fragment. (**A**) The fifteen most abundant mRNAs found in chimeric reads 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.

Figure 3.12

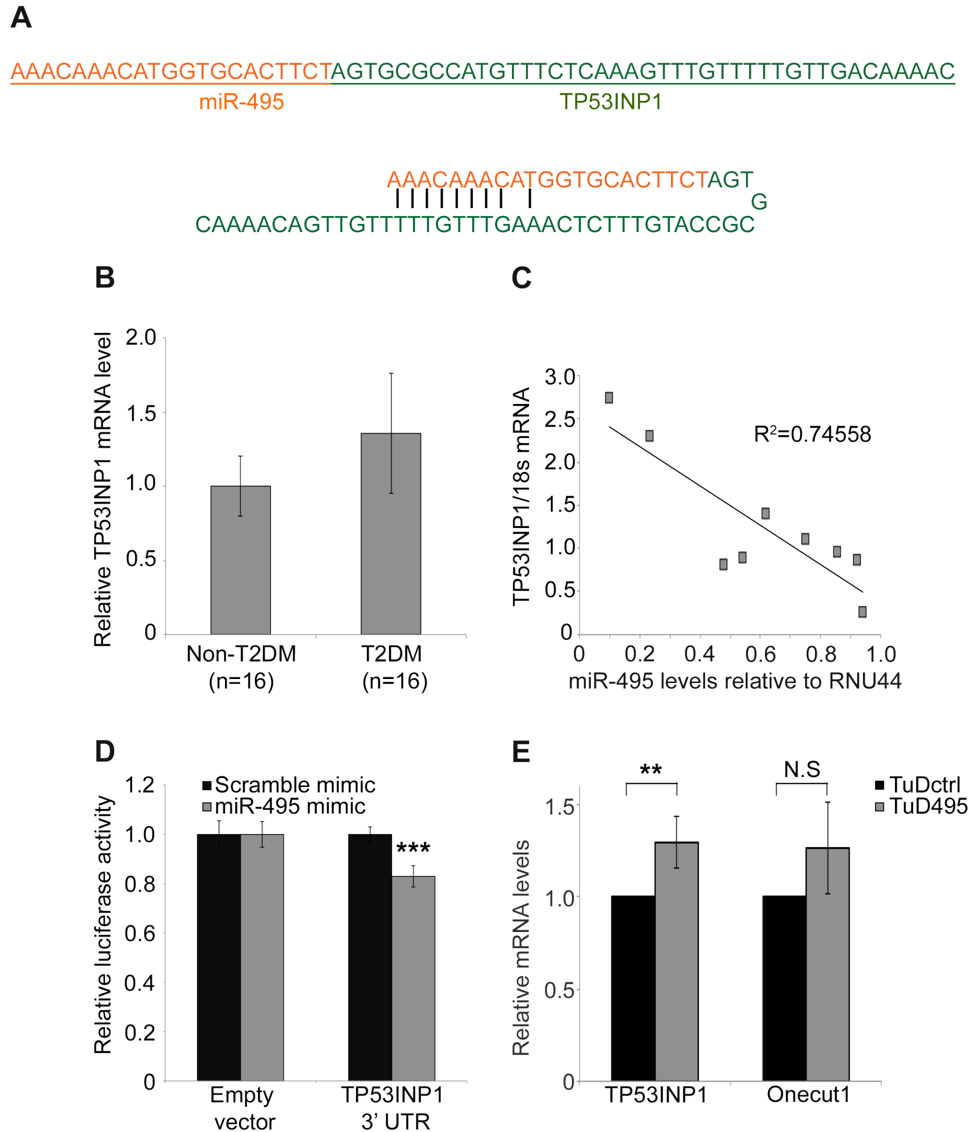

***Figure 3.12**: Validation of the miR-495:TP53INP1 targeting relationship*. A β-cell apoptotic factor, *TP53INP1* is regulated by miR-495. (**A**) The sequence of the miR-495 (orange) and *TP53INP1* 3'UTR (green) chimera. Folded confirmation with base pairing between the miRNA and 3'UTR is indicated 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 x 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.

Chapter 4

Regulation of the imprinted *DLK1-MEG3* locus through a distal β-cell enhancer

Abstract

Type 2 diabetes mellitus (T2DM) is a disease characterized by the inability of the insulin-producing β-cells to overcome insulin resistance in peripheral tissues. We previously identified an imprinted locus on Chr 14, the *DLK1-MEG3* locus, as being mis-regulated in T2DM human islets. However, very little is known about the mechanism by which imprinting at the *DLK1-MEG3* locus is regulated, particularly in human islets. Using targeted epigenetic modifiers, we prove that increased methylation at the promoter of *MEG3*, as observed in T2DM islets, results in decreased transcription of the maternal transcript. Additionally, we describe a novel enhancer in an intron of *MEG3*. In human islets, this enhancer is bound by transcription factors that are critical to islet function in an allele-specific manner. Using circular chromosome conformation capture followed by high-throughput sequencing, we demonstrate that the promoter of *MEG3* physically interacts with this novel enhancer and other putative regulatory elements in this imprinted region. Overall, our results suggest that the intronic *MEG3* enhancer plays an important role in the regulation of allelic expression at the *DLK1-MEG3* imprinted locus.

Introduction

Diabetes mellitus refers to a group of metabolic diseases characterized by an insufficient insulin response to high blood glucose levels. The pancreatic β-cells are critical regulators of glucose homeostasis as they produce, store and secrete insulin to regulate glucose uptake by peripheral tissues. Their autoimmune destruction or functional decline can lead to Type 1 and Type 2 diabetes mellitus (T2DM), respectively. Thus, understanding the molecular mechanisms underlying β-cell physiology is fundamental to improving current diabetes treatment strategies.

We previously demonstrated that the imprinted *DLK1-MEG3* locus is mis-regulated in islets from T2DM donors (Kameswaran et al. 2014). Genomic imprinting refers to the biased mono-allelic expression of genes in a parent-of-origin specific manner. The *DLK1-MEG3* locus consists of the paternally-derived *DLK1*, *RTL1* and *DIO3* genes, as well as maternally-expressed long non-coding RNAs, *MEG3*, *RTL1as* and *MEG8*, a large miRNA cluster and several snoRNAs (da Rocha et al. 2008; Charlier et al. 2001). The genes in this locus are very highly expressed in human β-cells (Dorrell et al. 2011; Kameswaran et al. 2014) and repressed in islets from T2DM donors (Kameswaran et al. 2014). This decreased expression correlates with hyper-methylation at the promoter of *MEG3*, which drives transcription of all the maternally-expressed genes in this locus. We uncovered targets of the microRNAs in this cluster that are relevant to diabetes pathogenesis, including pro-apoptotic genes *IAPP* and *TP53INP1* (Kameswaran et al. 2014). In addition, a risk variant for Type 1 diabetes was identified in an intron of *MEG3* in a genome-wide association study (Wallace et al. 2010). Consistent with these human studies, it has been reported that *Meg3* expression is decreased in mouse models of Type 1 and Type 2 diabetes, resulting in decreased insulin secretion and increased β-cell apoptosis (You et al. 2015). Overall, these observations suggest that the genes in the *DLK1-MEG3* locus are critical for β-cell health and function.

Despite its implication in diabetes and several cancers, very little is known about the mechanism by which imprinting at the *DLK1-MEG3* locus is regulated, particularly in human islets and β-cells. The monoallelic expression of the genes in this locus is established and maintained

through specific methylation patterns at two differentially methylated regions (DMRs), the germline-derived intergenic IG-DMR, located 13 kb upstream of the *MEG3* transcription start site, and the post-fertilization derived *MEG3*-DMR, overlapping the promoter of the maternal transcript (Schmidt et al. 2000; Takada et al. 2002). These DMRs are paternally-methylated, similar to another conserved imprinted locus, the *H19*-*Igf2* locus on mouse chromosome 7. In the *H19*-*Igf2* locus, this pattern of methylation at the imprinting control region results in differential access of downstream enhancer elements to the gene promoters and consequently, mono-allelic expression. Although the similarities between these imprinted loci have previously been observed (Wylie et al. 2000), the mechanism of imprinting at the *DLK1*-*MEG3* locus has not been described.

While *MEG3* promoter hyper-methylation and a concomitant decrease in expression has been reported in several human tumors and diseases, a causal relationship between these observations has not yet been established. Using targeted DNA methylation of the *Meg3*-DMR, we prove that hyper-methylation of this DMR causes decreased transcription of *Meg3*, consistent with our observation in islets from T2DM donors.

In embryonic stem cells, the maternal allele of the IG-DMR exhibits characteristics of an active enhancer that is transcribed to express short non-coding RNAs (Kota et al. 2014). Altering expression of these enhancer transcripts results in disruption of imprinted gene expression at the *Dlk1*-*Meg3* locus. However, the IG-DMR does not demonstrate enhancer-RNA like properties in mouse embryonic fibroblasts (MEFs) or other tested differentiated cells, suggesting a different mechanism of regulation in differentiated cells. From a recent global analysis of regulatory elements in human islets (Pasquali et al. 2014), we identified a putative enhancer within an intron of *MEG3* that is bound by transcription factors that are critical for islet function. We hypothesized that in human islets, this putative enhancer may play an important role in the regulation of allelic expression at the *DLK1*-*MEG3* locus, similar to the *H19*-*Igf2* paradigm (figure 4.1). We demonstrate that the intronic element at the *MEG3* locus is indeed and active enhancer, and is bound by islet transcription factors in an allele-specific manner. Finally, using circular

chromosome confirmation capture followed by high-throughput sequencing (4C-Seq), we demonstrate the long-range interactions of the *MEG3*-DMR and this intronic enhancer in human islets. Overall, our results suggest an important regulatory function for this newly characterized *MEG3* enhancer and provide insights into the mechanism of imprinting at the *DLK1*-*MEG3* locus in β-cells.

Results

Promoter methylation causes decreased expression of MEG3

We have previously demonstrated that the *MEG3* promoter is hyper-methylated in pancreatic islets from T2DM donors compared to non-diabetic donors, correlating with a decrease in expression of *MEG3* and its associated microRNAs (Kameswaran et al. 2014). To test whether increased methylation at this region directly causes a decrease in expression, we employed a promoter luciferase reporter assay. We sub-cloned the *MEG3* promoter region (*MEG3*-DMR) into a luciferase vector that lacks CpGs (pCpG-free) to avoid confounding signals resulting from methylation of the vector backbone. We employed the promoter of *CDKN2A*, which is known to be regulated by DNA methylation (Herman et al. 1995), as a positive control. These constructs were methylated *in vitro* using the CpG methyltransferase M.SssI. To confirm the successful *in vitro* methylation of the reporter plasmids, we utilized HpaII, a methylation-sensitive restriction enzyme whose recognition sequence includes a CpG. The vectors containing the *MEG3*-DMR and *CDKN2A* promoter were digested by HpaII in its unmethylated state, but were protected from digestion when methylated (figure 4.2A). As expected, a control vector without CpGs and hence that lacks a recognition site for the restriction enzyme, remained uncut regardless of its methylation status (figure 4.2A).

Upon transfection of the reporter plasmids into HeLa cells, we unexpectedly found that the unmethylated constructs no longer produced a robust luciferase signal. We observed similar results when the constructs were transfected into human HEK293T cells and βTC6 mouse insulinoma cells. To investigate whether this was an effect of the promoter sequences, we sub-cloned the same promoter regions into a standard luciferase reporter vector, pGL3. Unlike the pCpG-free vector, this backbone contains multiple CpGs and so even in its empty, unmethylated state, these constructs are cut by HpaII but are protected from digestion upon *in vitro* methylation, as predicted (figure 4.2A). When these pGL3 constructs were transfected into HeLa cells, we observed a 32- and 50-fold increase in luciferase activity by the unmethylated *MEG3*-DMR and *CDKN2A* promoter vectors, respectively, compared to the empty pGL3 vector. However, following

in vitro methylation their luciferase activities were comparable to the empty vector (figure 4.2B). Although these results are consistent with the dogma that increased methylation results in decreased expression, we cannot exclude the possibility that the decreased luciferase activity is a result of methylation of the pGL3 backbone rather than the promoter regions. Nevertheless, it confirms that it is the CpG-free vector backbone, and not the *MEG3* and *CDKN2A* promoter regions, that prevents promoter activity in the mammalian cell lines tested.

As an alternate approach to evaluate whether methylation of the *MEG3*-DMR directly causes decreased promoter activity, we employed customizable DNA binding proteins, called transcription activator-like effectors (TALE), that can target an effector molecule of interest, here DNA methyltransferases (DNMT), to a specific DNA sequence (D. L. Bernstein, Le Lay, et al. 2015; Sanjana et al. 2012) (schema outlined in figure 4.3). These TALE-DNMTs are efficient mediators of targeted DNA methylation (D. L. Bernstein, Le Lay, et al. 2015). We constructed a TALE-DNMT containing an EGFP marker that targets the mouse *Meg3*-DMR sequence 360 bp upstream of the *Meg3* transcription start site (TSS) (figure 4.4A). As a control, we introduced an inactivating point mutation into the DNMT catalytic domain (D. L. Bernstein, Le Lay, et al. 2015; J.-Y. Li et al. 2007). We transfected βTC6 mouse insulinoma cells and FACS-sorted cells that were successfully transfected based on their GFP expression. While the *Meg3*-DMR methylation levels across the regions tested were approximately 40% in the untransfected cells, the wildtype TALE GFP+ cells exhibited an increase in methylation of 20%, along with a 40% decrease in *Meg3* expression compared to untransfected cells (figures 4.4B and C). This effect was completely abolished in the mutant TALE GFP+ cells, verifying that the observed changes were specific to methyltransferase activity and not an artifact of transfection and FACS. To confirm that both transfected cell populations had received comparable amounts of TALE vector, we performed qPCR with primers specific to the TALE construct backbone (figure 4.4D).

It has previously been demonstrated that TALEs can have proximal off-targets effects (D. L. Bernstein, Le Lay, et al. 2015). To characterize possible non-specific targets of TALE binding and methyltransferase activity, we profiled DNA methylation at CpG islands near the *Meg3*-DMR.

The IG-DMR of the *Dlk1-Meg3* locus is located 16 kb upstream of the *Meg3* TSS and is also paternally methylated in both human and mouse β-cells (figure 4.5 and (Kameswaran et al. 2014)). However, we found this region to be fully methylated in untransfected βTC6 cells, as well as in WT and mutant TALE-DNMT transfected cells (figure 4.6A). Next, we tested the methylation levels of the *Dlk1* promoter, located 87 kb upstream of the *Meg3* TSS. Surprisingly, this region demonstrated a 20% increase in methylation in cells transfected with WT TALE-DNMT compared to cells transfected with the mutant construct (figure 4.6B-D). We observed no difference in methylation levels at two CpG islands close to the *Dio3* gene approximately 650 kb and 737 kb downstream of the *Meg3* TSS (figure 4.7).

In summary, using targeted epigenetic modifiers, we have shown that hypermethylation of the *Meg3* promoter causes its decreased expression, validating our observations in islets from T2DM donors.

Characterization of a novel enhancer in MEG3

From a recent study that profiled regulatory elements based on a comparative analysis of expression data, transcription factor binding networks and chromatin marks from human islet and FACS-sorted β-cells (Pasquali et al. 2014), we identified a putative enhancer within an intron of the *MEG3* gene. This region, located approximately 16 kb downstream of the *MEG3* TSS, is bound by transcription factors critical to islet development and function and marked by histone modifications that correlate with active enhancer activity, such as mono-methylation of histone H3 at lysine 4 (H3K4me1) and acetylation of histone 3 at lysine 27 (H3K27ac) (Creyghton et al. 2010) (figure 4.8A). Similar H3K27ac enrichment at this region has been observed in human lung fibroblast cell lines (NHLF), but not in other human cell lines tested (ENCODE Project Consortium 2012) (figure 4.8B). Two enhancers downstream of *H19* are critical for monoallelic gene expression at the *H19-Igf2* locus (figure 4.1) (Bartolomei et al. 1993; Leighton et al. 1995). Thus, we hypothesized that this putative intronic *MEG3* enhancer may be a critical regulator of mono-allelic expression at the *DLK1-MEG3* locus (figure 4.1).

First, we sought to validate the activity of the putative enhancer using luciferase reporter assays. We sub-cloned the *MEG3*-DMR into the pGL3 reporter vector and transfected this construct into βTC6 mouse insulinoma cells. The promoter sequence increased luciferase activity by 14-fold, validating its strong activity in β-cells (Kameswaran et al. 2014) (figure 4.9A). Addition of the intronic enhancer, in either orientation, further doubled luciferase activity (figure 4.9A). Additionally, we subcloned the enhancer sequence into the pGL4 luciferase vector that contains its own minimal promoter. The addition of the *MEG3* enhancer increased luciferase activity by five-fold over that of the minimal promoter construct (figure 4.9B). Thus, we confirmed that this intronic region of the *MEG3* gene indeed functions as an enhancer in β-cells.

Allele-specific transcription factor binding at the MEG3 enhancer

We have previously reported a dramatic down-regulation of *MEG3* expression in islets from T2DM donors compared to non-diabetic control islets (Kameswaran et al. 2014). We hypothesized that this difference may be caused, atleast in part, by decreased enhancer activity in T2DM islets, as a result of decreased transcription factor binding at the enhancer. To determine if transcription factor binding at the *MEG3* enhancer is differential between islets from T2DM and non-diabetic donors, we performed chromatin immunoprecipitation (ChIP) followed by qPCR (ChIP-PCR) using primers spanning the enhancer region. On average, we saw a six-fold enrichment of NKX2.2 binding at the *MEG3* enhancer in non-diabetic donors' islets compared to a 4.5-fold enrichment in islets from T2DM donors (figure 4.10A). However, it has previously been shown that expression of several islet-enriched transcription factors is down-regulated in T2DM (Guo et al. 2013). Therefore, we normalized our enrichment values to known binding sites of the specific transcription factor in question. For NKX2.2 ChIP, we used the *MAFB* enhancer sequence as a positive control. After normalization, we saw no difference in NKX2.2 enrichment between islets from six T2DM donors and eight non-diabetic donors (figure 4.10B). Thus, we conclude that occupancy of transcription factors such as NKX2.2 at this novel enhancer is similar

between islets obtained from non-diabetic and T2DM donors and is hence unlikely to contribute to the differential expression of the *MEG3* maternal transcript.

Allelic differences in chromosomal landscapes have been observed at imprinted loci such as the *H19*-*Igf2* locus (Verona et al. 2008). In mouse midgestation embryos, the open chromatin landscape of the *Meg3* promoter is restricted to the active maternal allele (Carr et al. 2007; Mcmurray and Schmidt 2012). Similarly, in MEFs, binding of the insulator protein CTCF to the *Meg3*-DMR is restricted to the maternal allele (Lin et al. 2011). To test whether the islet transcription factors that bind the *MEG3* enhancer are similarly restricted to a single allele, we performed allele-specific ChIP-PCR for FOXA2, NKX2.2 and PDX1. In order to differentiate the two alleles, we selected islets from donors who were heterozygous for rs3783355, a common single nucleotide polymorphism (SNP) that lies within the enhancer region (figure 4.8A). Importantly, this SNP does not overlap with the known consensus binding motifs for the three transcription factors being tested. Using islets from donors heterozygous for rs3783355, we performed ChIP-PCR and then high-throughput sequencing to determine the relative abundance of the two alleles quantitatively and with high confidence. We anticipated that the input material would have an equal representation of both alleles, while allele-specific transcription factor binding would result in a preferential amplification of a single allele (schema outlined in figure 4.11A). As predicted, we observed roughly equal number of reads for each allele in the input material for all samples, whereas the transcription factor binding was skewed towards one allele, with FOXA2 demonstrating the strongest allelic bias (figure 4.11B). As a control, we performed this assay on islets from donors who are homozygous for the major allele and found that both the input and FOXA2 ChIP samples predominantly displayed reads corresponding to the G allele, as expected (figure 4.11C). The presence of any A allele sequence reads indicates a low level of contamination in our samples.

Additionally, for an alternate, qualitative readout of allele-specific transcription factor binding, we took advantage of the fact that rs3783355 lies in the recognition site for a restriction enzyme, BanII. The major G allele is part of the recognition sequence for the enzyme, while the

minor A allele disrupts this sequence and prevents digestion by the restriction enzyme. Thus, following ChIP-PCR, we digested the PCR fragment with BanII. As expected, the input samples had both the uncut and digested fragments, corresponding to the A and G alleles, respectively. Conversely, the FOXA2 transcription factor ChIP samples primarily contained the digested (G allele) fragments with minimal uncut (A allele) fragments (figure 4.11D), indicating allele-specific occupancy by FOXA2.

These two methods independently verify that the islet transcription factors that bind the *MEG3* enhancer preferentially bind to a single allele. These results suggest that in human islets, the enhancer is regulated in an allele-specific manner.

Long-range interactions of the MEG3 promoter and enhancer

Based on the previously established role of enhancers in mediating imprinted gene expression and the observation that the *MEG3* enhancer is active and bound by important islet transcription factors in an allele-specific manner, we hypothesized that the *MEG3* enhancer may physically interact with the promoter to facilitate transcription of the maternal non-coding RNA transcript. To determine if there are any long-range interactions between the *MEG3* promoter and the newly characterized enhancer, we undertook circular chromosomal conformation capture followed by high-throughput sequencing (4C-Seq). This technique provides an unbiased sampling of all interacting partners of a selected region of interest (the "viewpoint"). We performed 4C-Seq using the *MEG3* promoter and enhancer as viewpoints separately, using human islets from two independent donors. This reciprocal approach allowed us to test the hypothesis that the *MEG3* promoter and enhancer interact with each other, while also identifying other chromatin interactions of these regulatory elements. To date, no information regarding the chromatin confirmation at the *DLK1-MEG3* locus has been reported in any tissue or species.

Consistent with our hypothesis, we found that the *MEG3* promoter displayed frequent interactions with the enhancer, and vice versa (figure 4.12 and 4.13). These results were reproducible between two biological replicates. Strikingly, the promoter and enhancer shared

many of their long-range interactions, including *DLK1*. Additionally, these regulatory elements made contacts with other putative enhancers within the imprinted domain, including an enhancer that lies intergenic to *DLK1* and *MEG3*, as well as an enhancer cluster downstream of *MEG3* that overlaps with the snoRNA transcript, *MEG8*. However, some interactions were unique to each viewpoint. One such example is the interaction between the *MEG3* promoter and an enhancer cluster upstream of *DLK1*. Thus, using 4C-Seq we have mapped the long-range interactions of the *MEG3* promoter and enhancer in human islets.

Discussion

The genes in the *DLK1-MEG3* locus have been implicated in a wide range of cancers (Benetatos et al. 2012) and human diseases, including Type 1 (Wallace et al. 2010) and Type 2 diabetes (Kameswaran et al. 2014). However, little is known about the regulation of imprinting at this locus. We describe here for the first time an intronic enhancer of *MEG3* that in human islets physically interacts with the promoters of *MEG3* and *DLK1*.

The *DLK1-MEG3* locus shares many similarities with another well-studied imprinted region, the *H19-Igf2* locus (Schmidt et al. 2000; Wylie et al. 2000). The latter consists of maternally expressed non-coding RNA, *H19* and miR-675, as well as a paternally expressed protein-coding gene, *Igf2*, located 90 kb apart and transcribed in the same direction. The mono-allelic expression of these genes is regulated by common regulatory elements, including a paternally methylated imprinting control region (ICR), and enhancers downstream of *H19* whose access to the individual genes is directed by differential binding of an insulator protein, CTCF, at the ICR (schema in figure 4.1). Unlike the insulator model of the *H19-Igf2* locus, the *DLK1-MEG3* locus contains a CTCF binding site at the *MEG3*-DMR rather than at the IG-DMR, the primary ICR of this region (Paulsen et al. 2001; Lin et al. 2011). In fact, CTCF occupancy at the *Meg3*-DMR is quantitatively comparable to that at the H19-ICR in MEFs (Lin et al. 2011). However, putative CTCF binding sites that were identified at the *MEG3*-DMR by ChIP-Seq in human islets (Stitzel et al. 2010) were not supported by subsequent CTCF human islet ChIP experiments (data not shown and (Pasquali et al. 2014)). Similarly, CTCF does not bind the *Meg3*-DMR in midgestation embryos (Carr et al. 2007). This discrepancy in CTCF occupancy suggests that it may function to regulate imprinting at the *DLK1-MEG3* locus in a different manner than at the *H19-Igf2* ICR. In support of this notion, CTCF has been reported to directly interact with RNAs, including *Meg3*, suggesting novel mechanisms by which this protein functions as a regulator of chromosomal interactions (Kung et al. 2015). Alternatively, allelic expression of genes in the *DLK1-MEG3* locus may be regulated in a tissue-specific manner. Indeed, only a few other cell types exhibit enrichment of H3K27ac, a marker of active enhancers (Creyghton et al. 2010), at

the *MEG3* enhancer, as we found in human pancreatic islets. Additionally, the combination of transcription factors shown to bind this enhancer are well-established regulators of β-cell maturation and function (Servitja and Ferrer 2004). Thus, the genes in the *DLK1-MEG3* imprinted locus may be regulated in a tissue-specific manner with the intronic *MEG3* enhancer playing an important role in their β-cell expression.

Allele-specific occupancy of transcription factors and histone marks has been demonstrated at *cis*-regulatory elements of several imprinted regions, including the *Meg3*-DMR (Carr et al. 2007; Mcmurray and Schmidt 2012; Verona et al. 2008). Our data suggest that the novel *MEG3* enhancer is also bound by islet transcription factors in an allele-specific manner. The allele-specific transcription factor occupancy at the *MEG3* enhancer in human islets suggests a model in which the enhancer is in open chromatin and active on the maternal allele, but silent on the paternal allele. However, because we could not obtain the parental genotypes for each islet donor, we were unable to determine if the transcription factor bound allele was indeed the maternal allele; all we could demonstrate was mono-allelic occupancy. To circumvent this problem, we attempted to take advantage of the well-established parental-specific methylation patterns at the *MEG3*-DMR. However, the enhancer and *MEG3*-DMR, which are located 16 kb apart, are unfortunately not in linkage disequilibrium, and thus we were unable to use the methylation levels of SNPs located in the DMR as a marker of the two parental alleles. Another distinguishing factor between the two alleles is the parental-specific expression of genes, but SNPs that are in linkage disequilibrium with the enhancer SNP rs3783355 did not overlap with exons of the maternally transcribed *MEG3*. Finally, we sought to repeat the allele-specific ChIP in islets from mice derived from a congenic mouse line (*Mus musculus castaneus* strain Cast/Ei chromosome 12 on *Mus musculus domesticus* strain C57BL/6 background), where the parental alleles can be determined definitively. Unfortunately, there were no strain specific polymorphisms in the conserved enhancer region to distinguish the alleles. Although we were unable to conclusively determine the parental allele to which the transcription factors were bound at the *MEG3* enhancer, we postulate that this occupancy must occur on the active maternal allele,

similar to what has been observed at the *Meg3* promoter in mouse (Carr et al. 2007; Mcmurray and Schmidt 2012).

We have previously reported that expression of *MEG3* and the associated miRNAs is lower in islets from T2DM donors, correlating with an increase in methylation at the promoter of this maternally-expressed non-coding RNA transcript (Kameswaran et al. 2014). While these results are consistent with the widely accepted dogma that increased methylation leads to decreased expression, it does not establish a causal link between these observations. A commonly utilized method to test the relationship between methylation and expression is treatment with the DNA demethylating agent, 5-aza-2-deoxycytidine (Yunli Zhou, Zhang, and Klibanski 2012). However, this drug acts globally to demethylate the entire genome, confounding data interpretation from such studies. To circumvent such issues, we employed TALE molecules fused to a DNA methyltransferase to specifically direct methylation to the *Meg3*-DMR in mouse β-cells. Using this targeted approach, we demonstrate that increased methylation of the *Meg3*-DMR in β-cells does in fact lead to a decrease in *Meg3* expression. We also observe an increase in methylation at the promoter of the paternally expressed *Dlk1*. While this change in *Dlk1* methylation levels may be attributed to unintended off-target effects of the TALE constructs, it is also conceivable that this is a result of the three-dimensional chromatin architecture of this region as we also demonstrate that the *DLK1* and *MEG3* promoters physically interact in human islets.

Overall, our results provide strong evidence for the role of a novel enhancer in the regulation of imprinting at the *DLK1-MEG3* locus in human cells. These results extend our understanding of allelic expression of genes in this important locus and thus its mis-regulation in T2DM and other diseases.

Figures

Figure 4.1

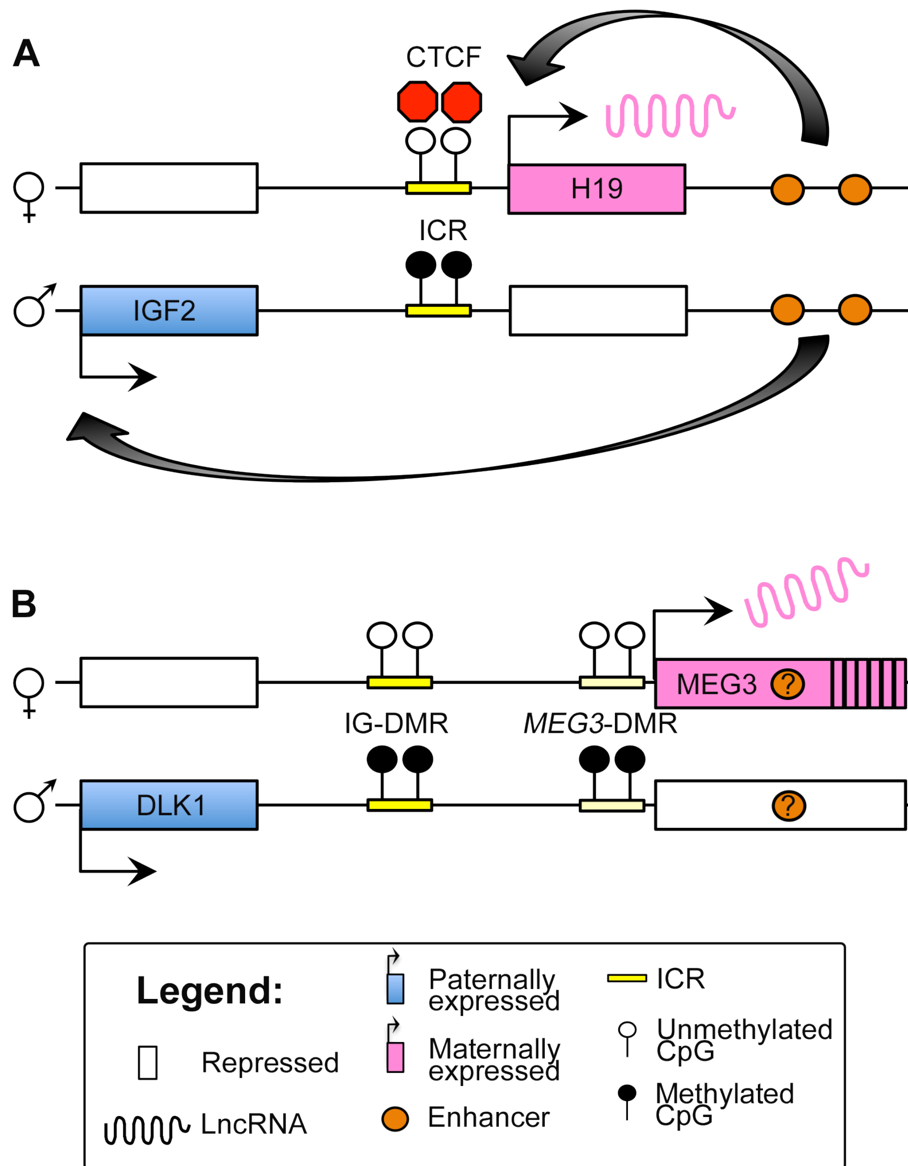

Figure 4.1: *Enhancers are critical regulators of mono-allelic expression at imprinted loci*. (**A**) Proposed model of imprinting at the *H19-Igf2* locus. Common enhancer elements downstream of *H19* differentially regulate transcription of *H19* and *Igf2* based on CTCF binding at the ICR. (**B**) Speculated model of mono-allelic expression in the *DLK1-MEG3* locus in human islets. A putative enhancer in an intron of *MEG3* is bound by critical islet transcription factors.

Figure 4.2

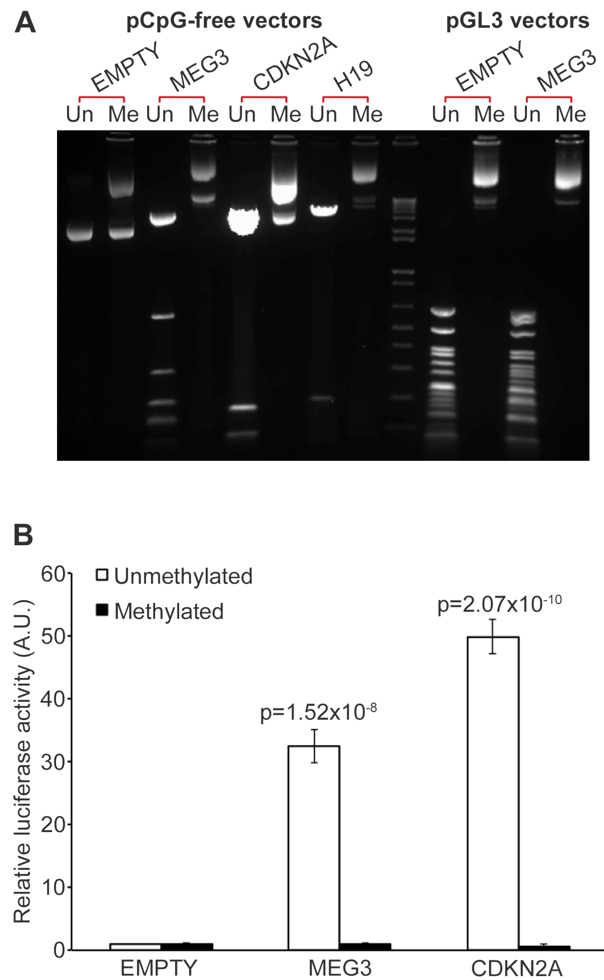

Figure 4.2: *Methylation-sensitive luciferase reporter assays.* **(A)** The *MEG3*-DMR, *CDKN2A* promoter and *H19* ICR were cloned into either pCpG-free or pGL3 luciferase reporter vectors. The plasmids were methylated *in vitro* with the M.SssI methyltransferase (Me) or left unmethylated (Un) and then digested with the methylation-sensitive restriction enzyme, HpaII. A representative gel of the HpaII digestion products is shown, indicating complete *in vitro* methylation of the reporter constructs. **(B)** Relative luciferase activity of pGL3 luciferase vectors containing the promoter sequences of *MEG3* or *CDKN2A* before and after *in vitro* methylation. The constructs were transfected into HeLa cells. *p*-value calculated using Student's t-test. n=3, error bars represent +/- SEM.

Figure 4.3

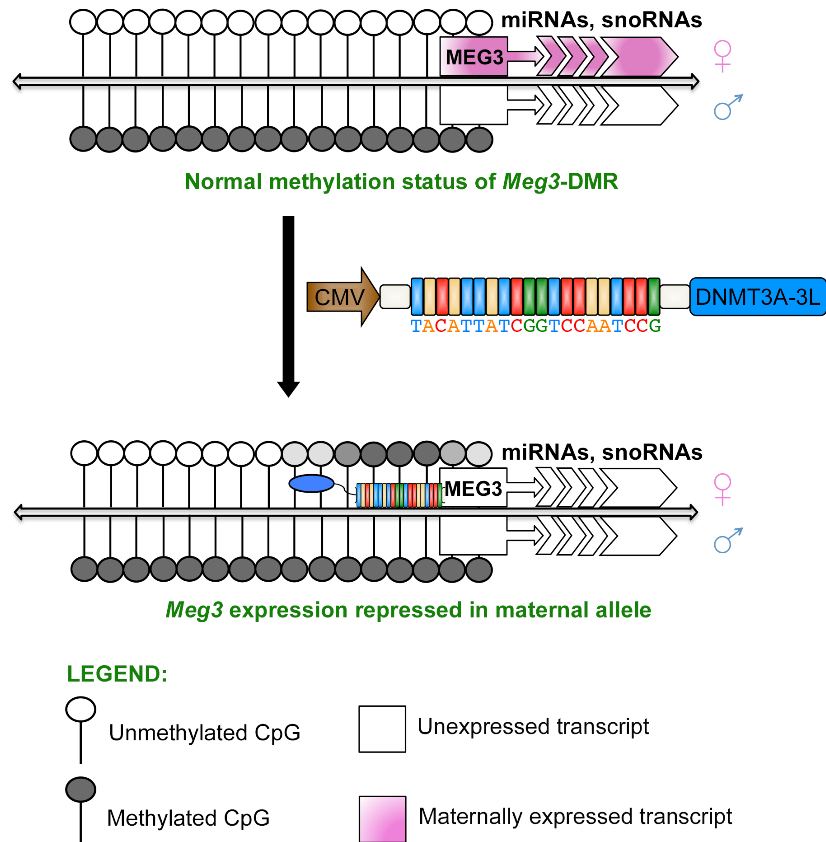

Figure 4.3: *Schematic representation of targeted methylation with TALE-DNMT constructs.* The *Meg3*-DMR is paternally methylated while the maternal allele is unmethylated with concomitant expression of the non-coding RNA transcript. Upon targeted recruitment of DNA methyltransferases using TALE molecules, we anticipate increased methylation of the CpGs on the maternal allele. We hypothesize that this increased methylation will result in repressed expression of *Meg3* and the associated small RNAs.

Figure 4.4

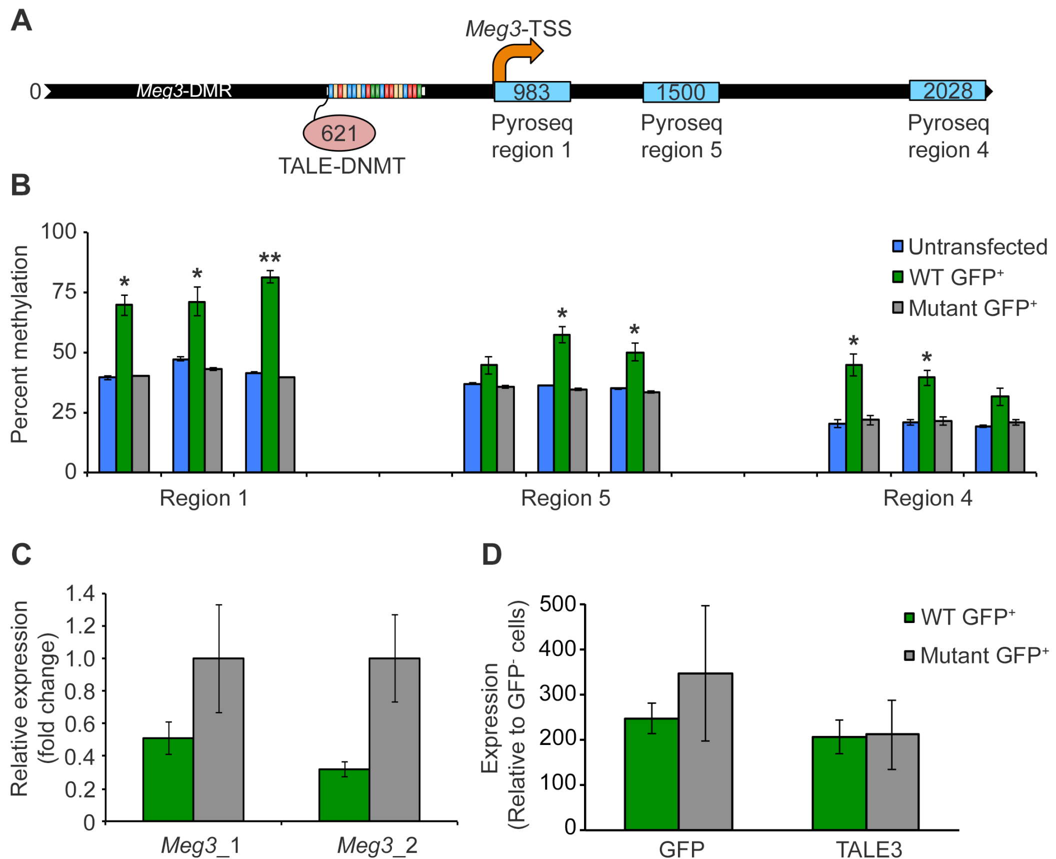

Figure 4.4: *Targeted methylation of the Meg3-DMR results in decreased expression.* βTC6 cells were transfected with TALE molecules specific to the mouse *Meg3*-DMR, fused to either a Wildtype (WT) or Mutant DNA methyltransferase catalytic domain. Transfected cells were sorted by GFP expression. **(A)** Schema of mouse *Meg3*-DMR depicting relative positions (bp) of TALE-DNMT binding, and *Meg3*-TSS. Regions assayed for methylation levels by pyrosequencing are depicted by blue boxes. **(B)** Percent methylation levels determined by pyrosequencing of CpGs in the *Meg3*-DMR in untransfected βTC6 (n=3), WT TALE-DNMT (n=3) or Mutant TALE-DNMT (n=2) transfected cells. **(C)** Relative expression of *Meg3* measured by RT-qPCR between WT (n=3) and Mutant (n=3) transfected cells. **(D)** Comparison of GFP and TALE backbone expression by RT-qPCR between WT (n=3) and Mutant (n=3) transfected cells. *p*-values calculated by Student's t-test *p<0.05, **p<0.001. Error bars represent +/- SEM.

Figure 4.5

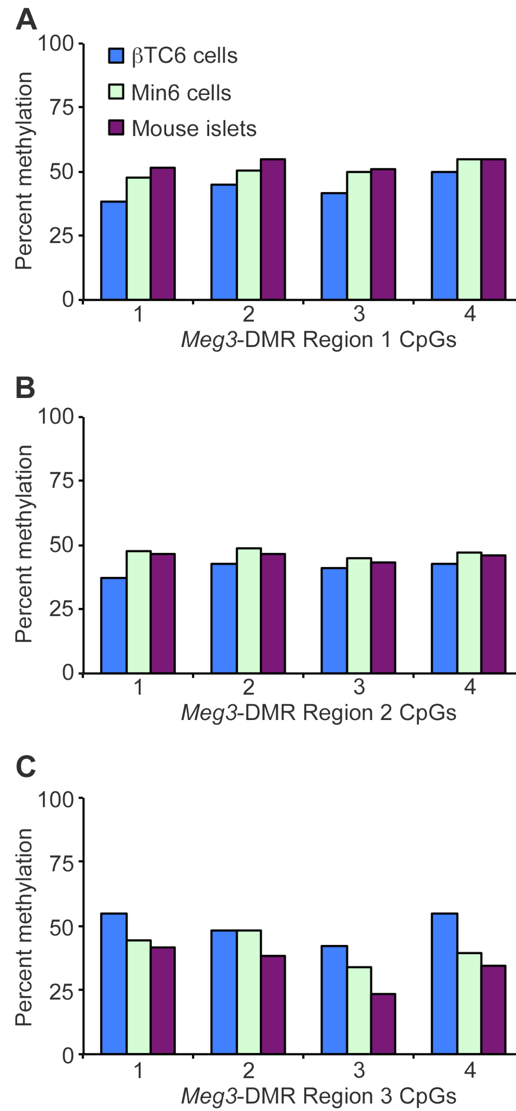

Figure 4.5: *The Meg3-DMR is imprinted in mouse β-cells.* Percent methylation of CpGs across the *Meg3*-DMR was determined by pyrosequencing of the mouse β-cell lines βTC6 and MIN6 as well as in islets from C57BL/6 mice.

Figure 4.6

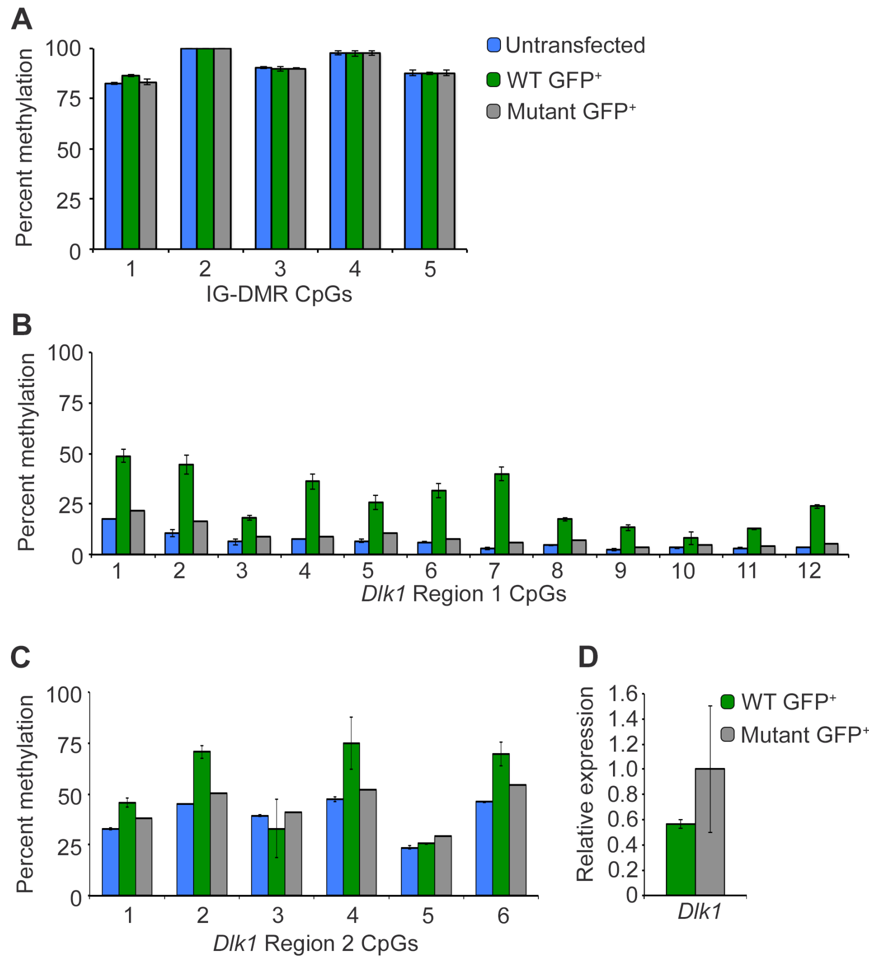

***Figure 4.6**: Targeted methylation of the Meg3-DMR results in increased Dlk1 methylation.* Methylation levels of CpG islands near the *Meg3*-DMR were assayed by pyrosequencing in untransfected βTC6 cells, as well as in WT GFP^{+} and Mutant GFP^{+} TALE-DNMT transfected cells. Percent methylation levels of CpGs in **(A)** IG-DMR (βTC6 cells (n=2), WT (n=3) and Mutant (n=3)). (**B**) *Dlk1* promoter region 1 (βTC6 cells (n=2), WT (n=3) and Mutant (n=1)). (**C**) *Dlk1* promoter region 2 (βTC6 cells (n=2), WT GFP^{+} (n=3) and Mutant GFP^{+} (n=1)). (**D**) *Dlk1* expression levels in WT (n=3) and Mutant (n=3) TALE-DNMT transfected cells. Error bars represent +/- SEM.

Figure 4.7

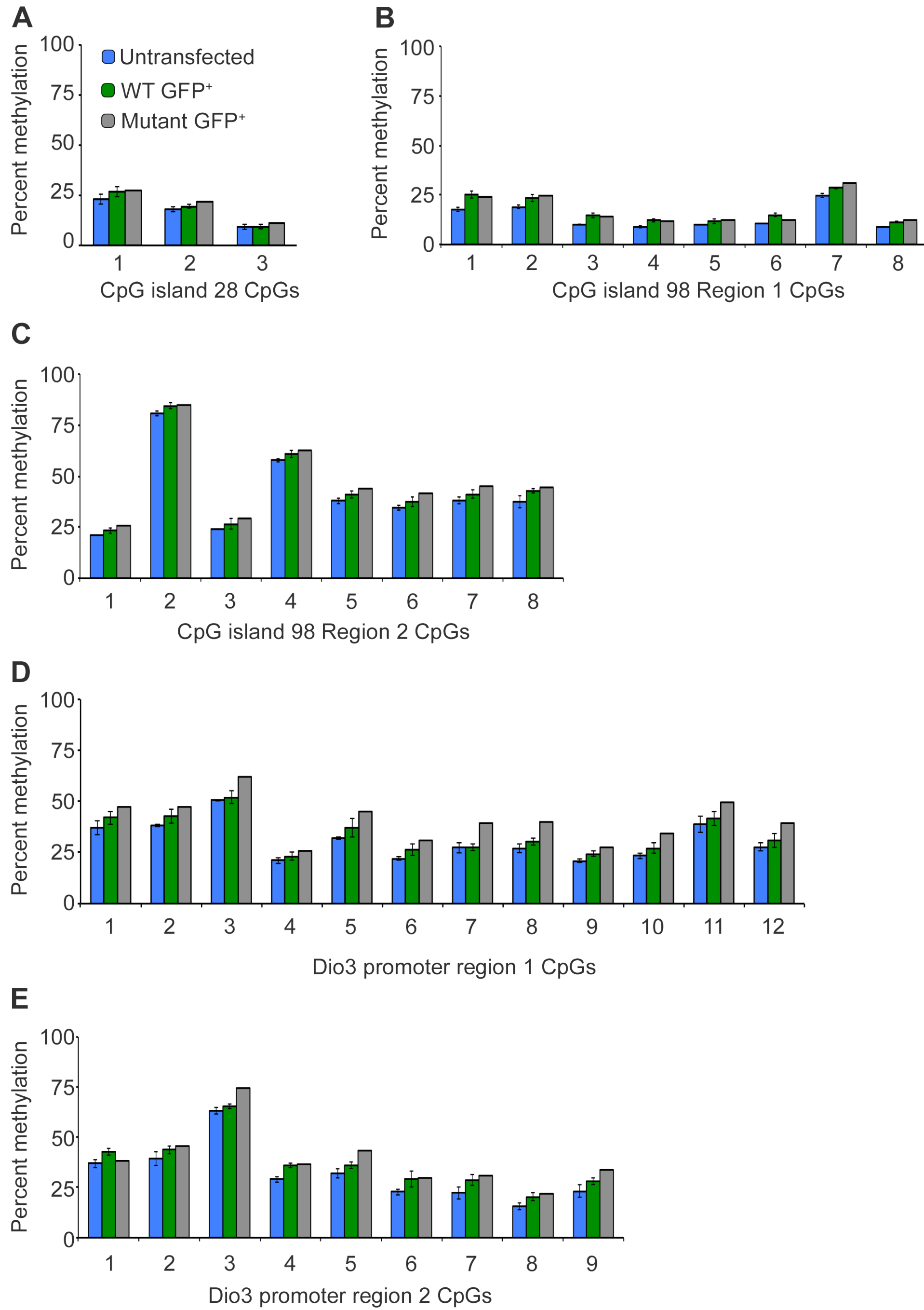

Figure 4.7: *Targeted methylation of the Meg3-DMR does not affect CpG islands downstream of Meg3.* Methylation levels of CpG islands downstream of the *Meg3*-DMR were assayed by pyrosequencing in untransfected βTC6 cells (n=2), as well as in WT GFP$^+$ (n=3) and Mutant

GFP^{+} (n=1) TALE-DNMT transfected cells. Percent methylation levels of CpGs in **(A)** CpG island 28 located approximately 650kb downstream of the *Meg3* TSS (**B**) CpG island 98 region 1 (**C**) CpG island 98 region 2 (**D**) *Dio3* promoter located approximately 730kb downstream of the *Meg3* TSS region 1 (**E**) *Dio3* promoter region 2. Error bars represent +/- SEM.

Figure 4.8

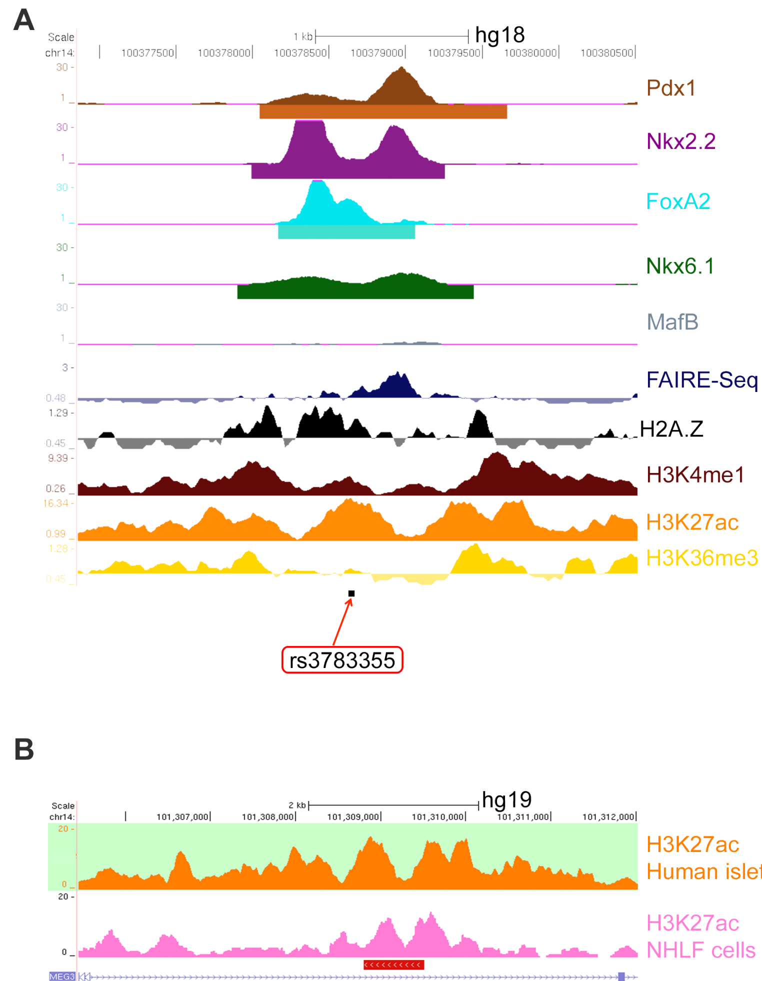

Figure 4.8: *Characteristics of a novel intronic enhancer human islets.* (**A**) The chromatin landscape for a putative enhance in an intron of *MEG3* is shown with human islet ChIP-Seq tracks for histone modification marks associated with enhancers (H3K4me1 and H3K27ac) and active transcription (H3K36me3), histone variant H2A.Z, and open chromatin regions identified by FAIRE-Seq. Occupancy of islet transcription factors Pdx1, Nkx2.2, FoxA2, Nkx6.1 and MafB at the putative enhancer is also shown. Data generated by (Pasquali et al. 2014). Relative position of the SNP rs3783355 is indicated by the black box. (**B**) Genome browser image of H3K27ac occurrence in human islets (Pasquali et al. 2014) and NHLF (ENCODE Project Consortium 2012) at an enhancer in the *MEG3* gene, depicted by a red bar.

Figure 4.9

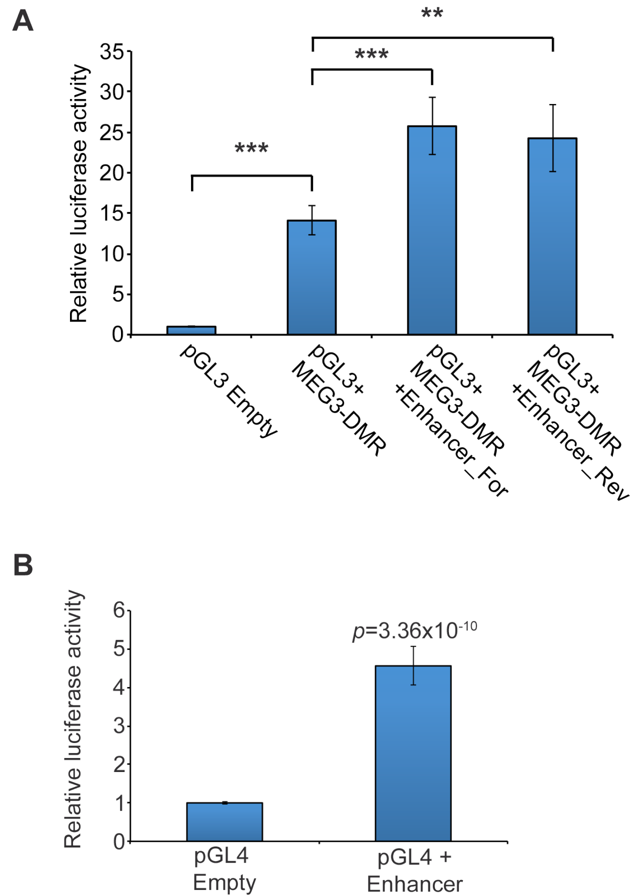

Figure 4.9: *A novel intronic enhancer in MEG3 is active in β-cells* (**A**) Activity of the *MEG3* enhancer was validated by luciferase reporter assays. pGL3 vectors with the *MEG3*-DMR and the enhancer sequence in either its native (For) or reverse (Rev) orientation were transfected into βTC6 cells. *p*-values calculated by Student's t-test. **$p<0.1x10^{-3}$, ***$p<0.1x10^{-5}$ n=3, Error bars represent +/- SEM. (**B**) Activity of the enhancer was evaluated using a luciferase reporter assay. The *MEG3* enhancer sequence was cloned into a pGL4 vector and transfected into βTC6 cells (n=4). *p*-value calculated using Student's t-test. Error bars represent +/- SEM.

Figure 4.10

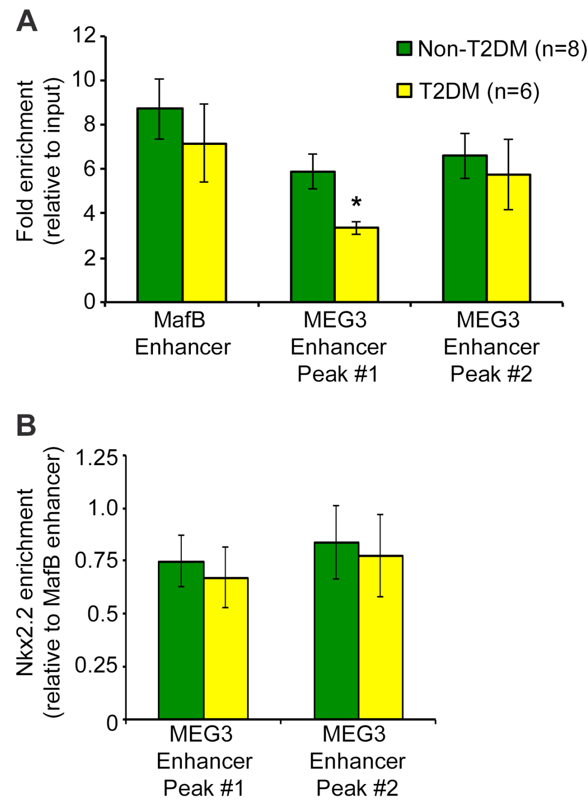

Figure 4.10: NKX2.2 *enrichment at the MEG3 enhancer is not different between islets from T2D and non-diabetic donors.* (**A**) NKX2.2 enrichment, relative to input, at the *MEG3* enhancer peaks determined by qPCR. The *MAFB* enhancer was used as a positive control. (**B**) NKX2.2 enrichment at the *MEG3* enhancer peaks, normalized to *MAFB* enhancer occupancy. *p*-values calculated by Student's t-test. *$p<0.05$. IP was performed using rabbit anti-NKX2.2 antibody (Sigma HPA003468) on islet chromatin from 8 non-diabetic and 6 T2DM donors. Error bars represent +/- SEM.

Figure 4.11

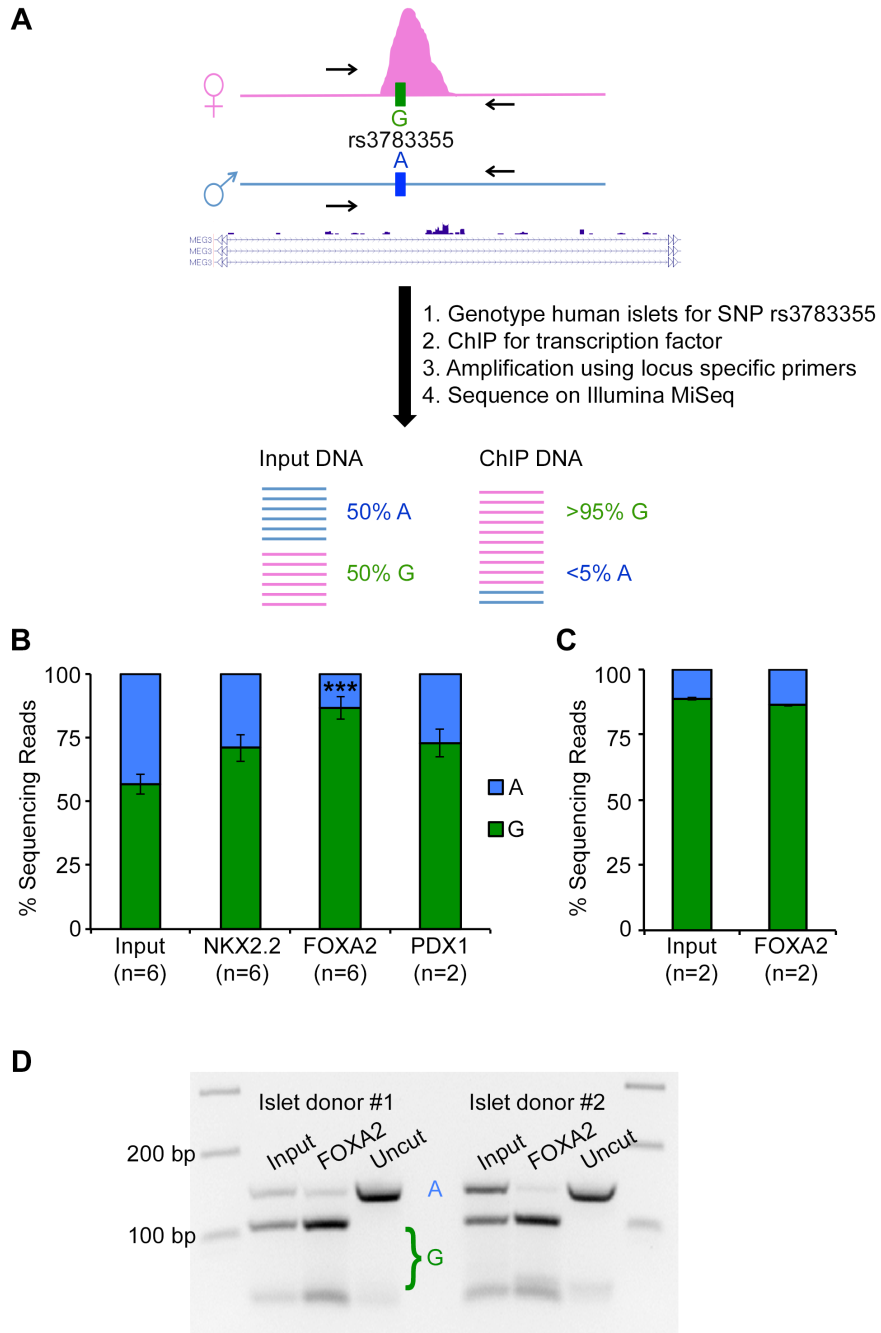

***Figure 4.11:** Allele-specific transcription factor occupancy at the MEG3 enhancer.* (**A**) Schematic representation of the allele-specific ChIP experimental design. Transcription factor ChIP is performed on islets from donors heterozygous for the SNP rs3783355. Following amplification

using primers surrounding the SNP, the PCR products are sequenced to quantitatively determine the relative representation of the two alleles. (**B**) Relative amplification (% sequencing reads) of the rs3783355 alleles as determined by high-throughput sequencing of input, NKX2.2, FOXA2 and PDX1 ChIP DNA from islet donors heterozygous for rs3783355 (G/A). (**C**) Relative amplification (% sequencing reads) of the rs3783355 alleles as determined by high-throughput sequencing of input and FOXA2 DNA from islet donors homozygous for the rs3783355 major allele (G/G). (**D**) The minor allele of rs3783355 alters the recognition sequence of a restriction enzyme, BanII. Following ChIP-PCR to determine FOXA2 occupancy at the *MEG3* enhancer, the PCR products were digested with BanII to qualitatively assess the allelic representation of rs3783355. A representative gel of BanII digested input and FOXA2 ChIP-PCR products from islets from two donors heterozygous for rs3783355 is shown. *p*-values calculated using Student's t-test, ***$p<0.001$. Error bars represent +/- SEM.

Figure 4.12

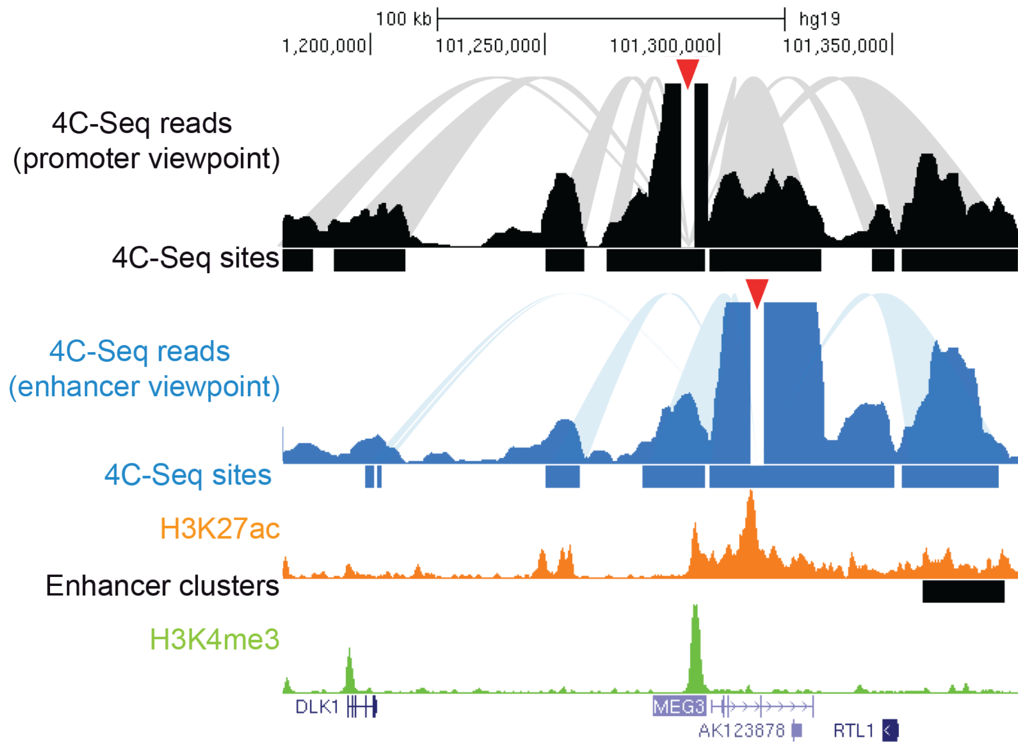

Figure 4.12: *Long-range interactions of the MEG3 promoter and enhancer identified by 4C-Seq in human islets*. Genome browser image of the selective interactions of the *MEG3* promoter and enhancer within 150 kb of the *MEG3* promoter. 4C-Seq was performed using the *MEG3* promoter and enhancer, respectively, as viewpoints (indicated as red triangles) using two human islet donors per viewpoint. Bars under each track represent the significant interaction sites (p<1x10-8). The *MEG3* promoter and enhancer make frequent contact with each other and also with DLK1 and a putative enhancer cluster downstream of *MEG3*. ChIP-Seq for the histone modification marks and the putative enhancer cluster identification was performed by (Pasquali et al. 2014).

Figure 4.13

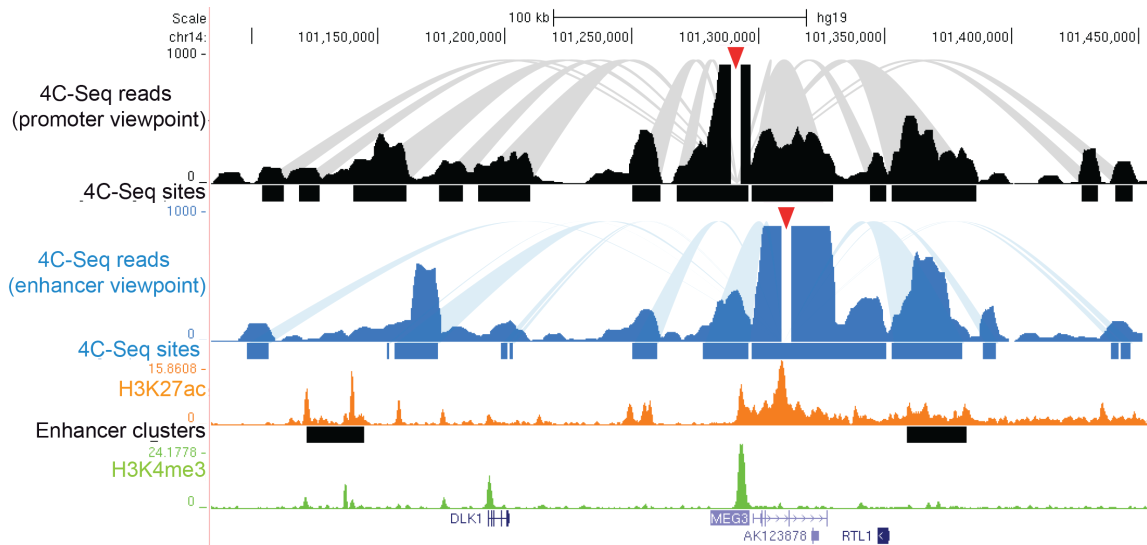

Figure 4.13: *The MEG3 promoter and enhancer interact with other putative enhancers within the DLK1-MEG3 imprinted region*. Genome browser image of the selective interactions of the *MEG3* promoter and enhancer within 350 kb of the *MEG3* promoter. 4C-Seq was performed using the *MEG3* promoter and enhancer, respectively, as viewpoints (indicated as red triangles) using two human islet donors per viewpoint. Bars under each track represent the significant interaction sites ($p<1x10^{-8}$). The *MEG3* promoter and enhancer make frequent contact with putative enhancers within the imprinted locus. ChIP-Seq for the histone modification marks and the putative enhancer identification was performed by (Pasquali et al. 2014).

Chapter 5

Discussion

In my thesis work, I have identified the *DLK1-MEG3* locus as an important genomic region that likely contributes to β-cell failure in diabetes. The genes in this locus are very highly and specifically expressed in human β-cells from the maternal allele. These genes are down-regulated in islets from T2DM donors, correlating with the hyper-methylation of their promoter. The miRNAs encoded by the *DLK1-MEG3* locus target disease-relevant genes such as *TP53INP1* and *IAPP*, whose activation can result in increased β-cell apoptosis. In addition to surveying expression of miRNAs in the *DLK1-MEG3* locus, I also characterized a novel intronic regulatory element in *MEG3* that functions as an enhancer in human islets and makes long-range interactions with multiple genes within this imprinted domain. Together, my data provide strong evidence for a role of miRNAs and epigenetic modifications, such as DNA methylation, in the pathogenesis of T2DM. These results are likely to have implications in multiple research areas and open new avenues of research, as I discuss below.

Expression of *DLK1-MEG3* genes in human islets

I have demonstrated that the miRNAs encoded within the *DLK1-MEG3* imprinted locus are specifically expressed in human β-cells, consistent with previous studies demonstrating β-cell specific expression of genes in this locus (Dorrell et al. 2011; van de Bunt et al. 2013). Using histone modification marks, I demonstrated that the promoter of *MEG3* drives expression of the entire maternal transcript in human β-cells, as had also been suggested for other cell types (Tierling et al. 2006; da Rocha et al. 2008). I found that the promoter of *MEG3*, which is highly expressed in β-cells, is marked by H3K4me3, a histone modification associated with active promoters. Conversely, in α-cells, where these genes are expressed at very low levels, the *MEG3* promoter is bivalently marked by both active and repressive histone modifications. This pattern of bivalent histone modifications is observed at genes that are silenced in embryonic stem (ES) cells but poised for activation at later stages of differentiation (B. E. Bernstein et al. 2006). Such bivalent domains have also been observed in genes differentially expressed between human α- and β-cells (Bramswig et al. 2013). In fact, many genes encoding critical regulators of β-cell

function are bivalently marked in α-cells. The discovery that the *MEG3* promoter displays a similar chromatin signature suggests that the genes under its control have a very specific role in β-cells and are disallowed in α-cells.

In line with my observation that a single promoter drives expression of the entire maternal non-coding RNA transcript, I found that the miRNAs encoded in this locus are all selectively expressed in β-cells and down-regulated in islets from T2DM donors. However, the expression levels of the different miRNAs are quite variable as observed by high-throughput sequencing and qRT-PCR. This expression data suggests that these miRNAs undergo post-transcriptional processing that affects their relative abundance. This variability may account for the fact that of the multiple miRNAs encoded in this region, only miR-127, the most abundant miRNA encoded by this locus, has previously been reported to be highly expressed in human islets (Bolmeson et al. 2011; Bravo-Egana et al. 2008; van de Bunt et al. 2013).

In my study, I did not observe differential gene expression of the miRNAs upon exposure of human islets to acute changes in glucose concentration. However, it was recently demonstrated that the murine *Meg3* gene expression is responsive to increasing glucose concentrations (You et al. 2015). The differing culture conditions and glucose concentrations used to conduct the studies may account for this discrepancy.

In summary, using chromatin signatures and transcriptional profiling, I have shown that the genes in this imprinted region are highly expressed in β-cells.

The *MEG3*-DMR is hyper-methylated in T2DM islets

I observed that in islets obtained from T2DM organ donors, the expression of the maternally-expressed miRNAs was repressed. This down-regulation is restricted to β-cells from T2DM donors rather than α-cells (unpublished data, Kaestner lab). As down-regulation of *MEG3* has been observed in many disease states and is often correlated with abnormal methylation of its promoter, I tested whether this was also the case in islets from T2DM donors. Using both qualitative (methylation-specific PCR) and quantitative (pyrosequencing) approaches, I

established that the decreased expression of maternal miRNAs in the *DLK1-MEG3* locus correlates with an increase in promoter methylation levels. While I found an increase in methylation of 15%, in reality this corresponds to an increase of 30% of the unmethylated allele as the quantitative measure of methylation that was used determines the average methylation across both alleles. This more pronounced difference is better captured by methylation-specific PCR, as this method allows for the visualization of differences between the methylated and unmethylated strands. More recently, *MEG3*-DMR hypermethylation in T2DM donor islets was validated by an alternative quantitative assay for DNA methylation, known as Bis-PCR[2] (D. L. Bernstein, Kameswaran, et al. 2015). This technique permits the more extensive coverage of CpGs in the *MEG3*-DMR compared to pyrosequencing and thus, we validated that the observed hyper-methylation extended across the entire DMR rather than at a few specific CpGs.

A fundamental limitation of my study is that the results of promoter hyper-methylation and concomitant decreased expression in human T2DM donor islets are correlative. To establish a causal link between these observations, I employed transcription activator–like effector molecules designed to target the *Meg3*-DMR, fused to a DNA methyltransferase catalytic domain (TALE-DNMT) (D. L. Bernstein, Le Lay, et al. 2015). Using this strategy, I proved that targeted methylation of the *Meg3* promoter results in decreased transcription of *Meg3* in a murine β-cell line. Thus, it is highly probable that the promoter hyper-methylation that we observe in the islets of T2DM donors precedes and causes the decreased transcription of the maternal non-coding RNAs.

These results add to the growing evidence that abnormal methylation patterns are observed at critical β-cell gene-regulatory regions in T2DM donor islets (Yang et al. 2011; Yang et al. 2012; Dayeh et al. 2014). However, with the use of islets from deceased T2DM donors as an experimental paradigm, it is impossible to distinguish whether these aberrant methylation events were the primary determinants of β-cell dysfunction in T2DM pathogenesis or a secondary consequence of the disease state. The use of model systems with controlled exposure to metabolic challenges will help to elucidate the order in which these events occur.

An intronic enhancer in *MEG3*

In my thesis work, I characterized a novel enhancer in an intron of *MEG3* that is bound by several key islet transcription factors in an allele-specific fashion. Several studies have now established that contrary to conventional belief, distal regulatory elements, rather than promoters, are drivers of islet-specific gene expression (Gaulton et al. 2010; Stitzel et al. 2010; Pasquali et al. 2014). However, the application of this information is challenging, as the target gene(s) regulated by these putative enhancers are not easily discernible. Therefore, I employed circular chromosome conformation capture followed by high-throughput sequencing (4C-Seq) to determine the long-range interactions of this enhancer in an unbiased manner. Using 4C-Seq, I demonstrated that the enhancer within the *MEG3* intron not only makes frequent interactions with the promoter of *MEG3*, but also with that of *DLK1* (located 100 kb upstream) and other distal enhancers, including an enhancer cluster located 70 kb downstream, overlapping *MEG8*. My data support the notion that transcription factor-bound enhancers function as *cis*-regulatory elements for distal target genes and underscore the utility of techniques such as 4C-seq in the unbiased identification of such interactions.

At present, apart from the presence of differential methylation patterns, very little is known about how allelic gene expression is regulated at the *DLK1*-*MEG3* locus. Despite efforts to identify potential *cis*-regulatory elements based on sequence similarity to the *H19*-*Igf2* locus, none of these putative enhancers were functionally validated (Wylie et al. 2000). In ES cells, the IG-DMR exhibits characteristics of an enhancer RNA that is bi-directionally transcribed, and lack of which results in altered expression of the genes in this locus (Kota et al. 2014). However, this region loses these features upon differentiation, suggesting that mature cells establish mono-allelic expression through different mechanisms. In my study, I present evidence for an active enhancer in primary human cells that makes long-range interactions with several genes within this imprinted domain. I demonstrate allele-specific occupancy of the bound transcription factors, as has been shown at the promoter of *Meg3* (Carr et al. 2007; Mcmurray and Schmidt 2012). The observation that the *MEG3*-DMR and transcription factor-bound enhancer interact suggests a

model whereby this chromatin looping facilitates transcription of the maternal genes. The increased methylation of the *MEG3* promoter observed in T2DM human islets would disrupt this interaction, resulting in the decreased expression of genes under the control of this promoter (figure 5.1).

Similarly, I show that the *MEG3* promoter makes frequent contacts with the paternally-expressed gene, *DLK1*. In support of this interaction, I observed that hypermethylation of the *MEG3* promoter using TALE-DNMT results in decreased expression of both *MEG3* and *DLK1*. Furthermore, *DLK1* expression is lower in T2DM β-cells, along with the maternally-expressed genes (unpublished data, Kaestner lab). The fact that the *MEG3* regulatory elements make physical contact with *DLK1* suggests that these interactions occur in in *trans* between the two parental chromosomes, or alternatively, that the *cis* interaction is constitutive on both alleles but gene expression is determined by other *trans*-acting determinants, such as transcription factor binding. It is plausible that other unidentified regulators contribute to the regulation of imprinting at this locus. Recent studies have suggested that similar to other nuclear lncRNAs, *MEG3* directly interacts with the PRC2 complex in ES cells to guide the repressive histone modification mark H3K27me3 to its target sites (Zhao et al. 2010; Kaneko et al. 2014). One study identified *Dlk1* as a direct target of the *Meg3*-PRC2 complex in mouse ES cells, although this finding could not be replicated in *MEG3*-expressing human iPSCs, where *MEG3* was found to function in *trans* (Kaneko et al. 2014). A careful characterization of *MEG3*-PRC2 complex targets in adult pancreatic islets will provide better insights into the role of this silencing complex in mediating allelic expression at this locus.

Future directions

In mid-gestation mouse embryos, active histone acetylation marks are present on the active maternal but not paternal allele of the *Meg3*-DMR (Carr et al. 2007; Mcmurray and Schmidt 2012). Whether such allele-specific histone patterns exist in the human *MEG3*-DMR can be determined using the allele-specific ChIP experimental model that we applied for the enhancer

transcription factors in Chapter 4. However, unlike in the instance of the enhancer, we can establish definitively the parental alleles using the methylation pattern of CpGs in the DMR as a surrogate.

While we demonstrate novel long-range interactions of the *MEG3* enhancer in human islets, their relevance to the repressed expression of genes in this locus in T2DM donor islets remains to be determined. Thus, we would like to characterize the chromosomal architecture of the *MEG3* enhancer in islets from T2DM donors. However, 4C-Seq generates a qualitative readout of chromosomal interactions and hence may not be sensitive to subtle differences in such interactions. For this reason, we believe that chromosome conformation capture followed by quantitative Taqman PCR (3C-qPCR) is a more suitable assay to quantitatively determine differences in the chromosomal landscape of islets obtained from T2DM and non-diabetic donors. While studies in human islets will help establish a correlation between the *MEG3*-DMR methylation status and chromosomal looping of the enhancer, studies in model systems are required for proving causality. For this reason, we plan to take advantage of TALE-DNMT constructs designed to this region, as the targeted methylation of the *MEG3* promoter will allow for the direct interrogation of its consequences on chromosomal architecture. However, an outstanding challenge of the application of TALE-DNMTs is the efficient delivery of these molecules to large quantities of human islets, as examining chromosomal interactions requires large amounts of starting material to robustly capture rare interaction events. Ongoing efforts to improve viral transduction efficiency include the disassociation of islets prior to infection, followed by reaggregation of the transduced cells. Alternatively, TALE transduction of the human β-cell line, EndoC-βH1, may enable these studies as these cells recapitulate many, but not all, features of human β-cells (Ravassard et al. 2011).

Human chromosome 14q32 shares synteny with the distal part of mouse chromosome 12, including the *DLK1*-*MEG3* locus. Importantly, the intronic *Meg3* enhancer exhibits strong sequence similarity to the human enhancer, with near-perfect conservation of the regions occupied by the transcription factors. We have derived mice in which loxP sites flank the

enhancer to permit its conditional ablation in β-cells. This mouse model will enable us to thoroughly evaluate the effect of disruption of this regulatory element on a molecular and physiological level.

Overall, my results improve our current understanding of gene regulation at the *DLK1-MEG3* imprinted locus in human islets. Additionally, I illustrate the importance of a novel β-cell enhancer, thereby providing a model with which to interrogate these putative regulatory elements in human islets. *MEG3* is expressed in several human tissues and its down-regulation is observed in many disease states. Thus, my studies will likely benefit many fields.

The human islet miRNA repertoire

Despite their implication in various aspects of glucose homeostasis and diabetes pathogenesis, the miRNA transcriptome of islets from T2DM donors had never been determined. In our study, we employed several high-throughput and unbiased techniques to thoroughly profile the human islet miRNA transcriptome as well their targets. First, we examined the miRNA expression in islets from non-diabetic and T2DM donors using small-RNA sequencing. We then performed a comparative analysis between these two groups to identify miRNAs that are differentially expressed in the T2DM disease state. Furthermore, using high-throughput sequencing following cross-linking and immunoprecipitation (HITS-CLIP), we identified Argonaute-associated miRNAs and their targets in human islets. Collectively, these datasets are a valuable resource for T2DM miRNA research.

Perhaps the most unexpected result from the HITS-CLIP analysis was the identification of chimeric reads, i.e. miRNAs that are intramolecularly ligated to their target mRNAs while in the complex with Argonaute. These reads are a direct source of mRNA:miRNA targeting relationships and thus obviate the need for prediction algorithms entirely. Although these reads make up a small fraction of sequences obtained from HITS-CLIP, we identified several informative miRNA-target pairs. These include miR-495, a miRNA encoded in the *DLK1-MEG3* locus, and its target

TP53INP1, a gene associated with a risk variant for T2DM by GWAS (Voight et al. 2010). Using luciferase reporter assays and miRNA 'tough decoys' (Haraguchi, Ozaki, and Iba 2009), we validated this miRNA-target interaction as true and robust. In addition, we assessed the relative RISC association, an indicator of functional relevance of miRNAs in such chimeras (Schug et al. 2013). The value of such reads has been recognized in other CLIP-based studies (Kudla et al. 2011) and inspired the development of CLASH, a technique which promotes this proximal ligation (Helwak et al. 2013). In CLASH, the incorporation of an intra-molecular ligation step increases the fraction of chimeric reads from 0.1 to 2%. From our HITS-CLIP and chimeric reads analysis, we identified that miRNA targeting are not restricted to the 3'UTR of mRNAs alone, with many target sites located in the coding sequence. These data corroborate the findings of other HITS-CLIP and CLASH datasets (McKenna et al. 2010; Chi et al. 2009; Helwak et al. 2013). This study highlights the benefit of *in vivo*, rather than *in silico* miRNA target identification.

Future directions

Using a wide range of high-throughput techniques, I have generated a catalog of miRNAs and their targets that are relevant to T2DM pathogenesis, as I described in Chapter 3. The identification of other *in vivo* miRNA-target combinations by CLASH may expand this important resource.

I have established that the miRNAs in the *DLK1-MEG3* locus are down-regulated in islets from T2DM donors. However, the functional consequences of loss of either individual or al miRNAs in this cluster on β-cells are unknown. As the maternal RNAs are transcribed from a common promoter, manipulating this DMR would result in the mis-expression of all the RNAs encoded by this locus, and also affect genes regulated by long-range chromosomal interactions with this promoter, thus confounding the interpretation of any phenotype. For this reason, we derived a second mouse line in which miR-127 and miR-136 are flanked by loxP sites to enable the β-cell-specific ablation of these miRNAs selectively. We expect that disruption of these miRNAs might affect the transcription and/or processing of the other miRNAs encoded in this

transcript, but not of *Meg3*, which is upstream. miR-127 is the most abundantly expressed miRNA in this locus and in humans, its expression was found to negatively correlate with insulin secretion (Bolmeson et al. 2011). miR-127 and miR-136 are believed to target the antisense paternally-expressed gene *Rtl1* by RNAi-mediated silencing due to their perfect complementarity (Seitz et al. 2003). We have revealed that other miRNAs encoded in this cluster target the pro-apoptotic factors *TP53INP1* and *IAPP*, and we therefore predict that the repression of these miRNAs will activate *TP53INP1* and *IAPP* and result in increased β-cell death. Thus, the conditional ablation of these miRNAs in β-cells will recapitulate their repression in T2DM donor islets and allow for the verification of these and other candidate targets, as well as the analysis of their functional consequences.

Although we focused our attention on the *DLK1-MEG3* imprinted miRNAs, other miRNAs identified through this study warrant further investigation. One such miRNA is miR-187, which we and others identified to be up-regulated in islets from T2DM donors (Locke et al. 2014). The expression of this miRNA inversely correlates with glucose stimulated insulin secretion. Thus, further investigation of its target mRNAs and their functions will improve our understanding of the relevance of this miRNA to T2DM pathogenesis.

In summary, this body of work cataloged human islet microRNAs relevant to T2DM pathogenesis and characterized their target transcriptomes. While studies in model systems provided a strong basis to consider miRNAs as likely candidates for T2DM development, none had directly profiled islets from T2DM human donors. While we were able to validate some of the miRNAs that rodent and *in vitro* models implicated in T2DM most were not differentially expressed between human islets from T2DM and non-diabetic donors. Although this does not explicitly demonstrate that these miRNAs are not relevant to β-cell function, it does highlight the general disadvantage of relying solely on model systems to identify functional candidates for human diseases. The typical model for implicating any miRNA in T2DM pathogenesis involves the characterization of a single candidate target gene through which it might function, usually identified through computational algorithms. The targeting relationship between the miRNA and

mRNA is often demonstrated by manipulating expression of the miRNA *in vitro* to non-physiological levels. This approach is generally misleading, as miRNAs regulate multiple genes simultaneously, predominantly by fine-tuning gene expression (Ebert and Sharp 2012), and thus selective elucidation of a single target in such a manner does not accurately represent the significance of any given miRNA to a biological process. The human islet HITS-CLIP and chimeric analysis data sets generated in this study will serve as a valuable resource to identify the group of targets that any miRNA may regulate *in vivo* and thus provide a better platform to characterize miRNAs that are relevant to T2DM pathogenesis.

Figures

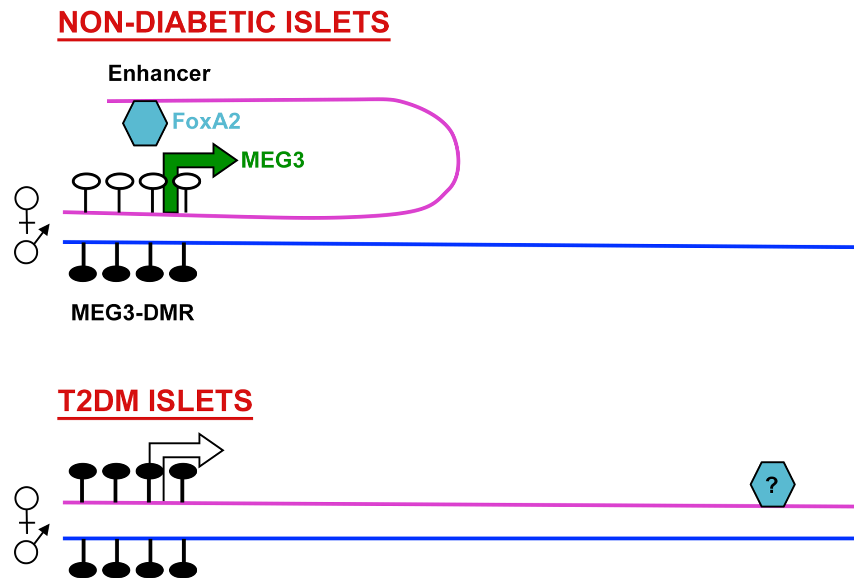

***Figure 5.1**:* Proposed model of *MEG3* enhancer-promoter interaction. In islets from non-diabetic organ donors, the enhancer interacts with the *MEG3*-DMR, which is unmethylated on the maternal allele and methylated on the silenced, paternal allele. This interaction may be mediated by allele-specific binding of transcription factors such as FOXA2. However, this interaction may be disrupted in T2DM islets, where the promoter is hypermethylated.

Chapter 6

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