MicroRNA-mediated regulation of Cadm1 contributes to beta cell function and energy homeostasis

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1 Introduction

1.1 Pathogenesis of Diabetes Mellitus

1.1.1 The History of Diabetes

Diabetes mellitus is a chronic condition, which is characterized by elevated blood glucose levels termed as hyperglycemia. Driven by dramatic changes in life style within the past 30 years, diabetes has been emerged as a pandemic and constitutes a major social and economic burden in developing and developed nations (van Dieren et al., 2010). Globally, about 350 million people suffer from diabetes that resulted in 3.4 million deaths due to consequences of high blood sugar in 2004 alone (Danaei et al., 2011). Chronic hyperglycemia often leads to damage of blood vessels, nerves, eyes and kidney and increases the risk for heart diseases and stroke. Diabetes is linked to polyuria and loss of body weight and first described by ancient Egyptians around 1500 BCE (Polonsky, 2012). However, only by the end of the 19th century Joseph von Mering and Oskar Minkowski discovered in dogs that the pancreas was involved in the regulation of blood glucose concentrations (Keck and Pfeiffer, 1989). Frederick Banting and his student assistant Charles Best ultimately discovered insulin in 1921 and could successfully treat diabetes in dogs (Banting and Best, 1990). This approach was translated to humans for the first time in 1922, where isolated bovine insulin was used to normalize blood glucose levels of a diabetic patient (Polonsky, 2012). This medical feat was subsequently rewarded with the Nobel Prize in Medicine in 1923. In addition to its fundamental role in the regulation of glucose homeostasis, insulin also led to many advances in science, resulting in a total of seven Nobel Prizes. This includes the 1958 Nobel Prize in Chemistry for deciphering the protein sequence of insulin by Frederick Sanger and the 1977 Nobel Prize in Medicine for the development of radioimmunoassays for insulin by Rosalyn Yalow.

1.1.2 Molecular Mechanisms contributing to the development of Diabetes

Diabetes is a very complex and heterogeneous disease that often features a strong genetic association together with changes in life style. Though hyperglycemia is a common feature of diabetes, the involved molecular mechanism and the consequent treatment differs dramatically. In addition to a few rare forms of the disease, there are two major types of diabetes. Type 1 Diabetes (T1D) constitutes about 5 to 10 % of all

cases and affects mainly young people as a result of autoimmune destruction of the insulin producing beta cells. On the other hand, Type 2 Diabetes (T2D) accounts for the majority of patients and is more common in the elderly, but nowadays also affects young adults due to dramatic changes in life style (Polonsky, 2012). Although there are rare monogenic forms of T2D known, most cases are caused by a wide range of genetic variations and environmental factors (Doria et al., 2008). Recently, alterations in the gut microbiome have also been implicated as a risk factor for the development of T2D (Khan et al., 2014). One of the main causes for T2D is insulin resistance, a state where peripheral insulin responsive tissues such as the liver, muscle and fat, fail to respond to insulin. Subsequently, gluconeogenesis is not suppressed in the liver and glucose is not taken up by muscle and fat. However, insulin resistance does not necessarily result in T2D as the pancreatic islet can compensate for the relative deficiency of insulin. In later stages beta cell failure occurs and leads to the progression of T2D (Schwartz et al., 2013).

1.1.2.1 Genetic contributions of Type 2 Diabetes

For the past several years, the genetic architecture has been considered as the main cause of the pathogenesis of T2D. However, only recent advances in sequencing technology within the last decade have opened the gate for genome wide association studies (GWAS). These studies compare up to thousands of individuals and allow the identification of genetic variants that correlate with the disease. To date about 65 variants have been identified, however these variants contribute to only 10 % of familial aggregation of the disease and increase the risk of T2D by 10 to 30 % (Ahlqvist et al., 2011; Morris et al., 2012). Interestingly, the majority of these variants are located in non-coding regions, which makes it difficult to study the biological function. A very recent investigation emphasizes this problem. Genetic variants within the gene *Fto* have been previously been shown to be associated with obesity and T2D. Moreover, changes in expression of *Fto* also correlate with obesity. However, the identified genetic variants are not associated with changes in *Fto* expression. Interestingly, association studies revealed a direct interaction with the gene *Irx3* in mega base distances. IRX3 is a regulator of body weight and metabolic rate and its expression correlates with genetic variants within the *Fto* gene (Smemo et al., 2014). Taken together, genetic studies have helped to identify multiple genetic risk loci that strongly correlate with the pathogenesis

of T2D. However, the functional contribution is not well understood and requires a thorough investigation.

1.1.2.2 Environmental Factors and Life style

While genetics attributes to about 30 to 70 % of the risk to develop T2D, environmental factors are considered to be another major driver of the pathogenesis of diabetes (Doria et al., 2008). Changes in life style within the past 50 years, have contributed as major risk factors for the development of T2D. Imbalanced intake and consumption of food, paired with poor quality constitutes a major driver. Physical inactivity further amplifies misbalance and synergistically results in overweight and obesity, which is the single most prominent predictor of T2D (Hu et al., 2001). In Germany alone, about two third of the male adult population is over-weight (Body Mass Index (BMI) of $\geq$ 25 kg/m^2), while 23 % are obese (BMI of $\geq$ 30 kg/m^2) (Mensink et al., 2013). Obesity is characterized by a dramatic accumulation of fat in the body. As a consequence, free fatty acids (FFA) accumulate in the plasma and inhibit the insulin-signaling pathway in muscle and liver (Perry et al., 2014a). Furthermore, insulin resistance results in a reduction of glucose uptake in muscle and diminished suppression of gluconeogenesis in the liver. Other risk factors for the development of T2D include aging and lately, intrauterine environment has been also implicated in the prevalence of diabetes (Doria et al., 2008).

1.1.3 Mechanisms of Glucose Homeostasis

Genetic factors as well as changes in lifestyle constitute a main cause in the development of diabetes. However, we are far away from understanding how these factors cause the disease. Most of these factors are involved in insulin secretion, insulin resistance and obesity, further underling that glucose homeostasis is maintained by a complex interplay between multiple tissues (Ahlqvist et al., 2011). Despite varying intervals of feeding and fasting in mammals, blood glucose levels under normal circumstances remain within a narrow range of 4.5 - 6.5 mM. This is accomplished by a tight coordination of multiple tissues and involves the uptake of glucose from the intestine, the production of glucose by the liver as well as the uptake of circulating glucose by peripheral tissues (Saltiel and Kahn, 2001). Normally, the blood glucose levels rise after ingestion, due to a rapid absorption of glucose by the sodium-glucose-transporter SGLT1 within the small intestine (Wright et al., 1992). This increase in

glucose is sensed by beta cells of the pancreatic islets and results in the release of insulin into the blood stream. Insulin mediates two different processes that contribute to lower glucose levels. First, it suppresses gluconeogenesis in the liver and secondly it mediates insulin-dependent glucose uptake in peripheral tissues such as the skeletal muscle and fat tissue. Here, the activated insulin-signaling pathway results in uptake of insulin due to the translocation of the Glut4 transporter to the plasma membrane. Furthermore, the brain also utilizes glucose in an insulin-independent manner. During fasting, plasma glucose levels slowly decrease, which stimulates the release of glucagon from pancreatic alpha cells. Glucagon activates the production and release of glucose from the liver by activating key enzymes like Fructose-bisphosphatase 2, which switches from glycolysis to gluconeogenesis and ultimately contributes to an increase of blood glucose level (Kurland et al., 1992). In addition to islet-released hormones, several other factors contribute to the regulation of glucose homeostasis. For instance, the adipocytes release free fatty acids, which reduce inulin sensitivity in liver and muscle, but also diminish glucose stimulated insulin release from the beta cell (Bergman and Ader, 2000). Furthermore, adipocytes release various hormones and other factors, which regulate the function of the brain and other tissues (Waki and Tontonoz, 2007). The satiety hormone leptin is one of the well-characterized adipokines that was first identified by Friedman and colleagues (Zhang et al., 1994). The main site of leptin action is the hypothalamus in the brain, where it regulates food intake, energy expenditure, and insulin sensitivity (Grayson et al., 2013). In addition to these metabolically relevant tissues, the gut has long been identified as another major source of hormones such as the Glucagon-like peptide-1 (GLP-1) (Gromada et al., 1998; Holst, 2007). This peptide hormone is specifically released by the intestine and stimulates the release of insulin from the pancreatic beta cell. Moreover, studies have established that it improves insulin sensitivity and suppresses appetite (Donnelly, 2012). Another example is ghrelin, a peptide hormone secreted by the stomach. Ghrelin antagonizes leptin action in the brain and increases appetite and adiposity (Yi and Tschop, 2012). Taken together, multiple hormones derived from different tissues contribute to the maintenance of glucose homeostasis (Figure 1).

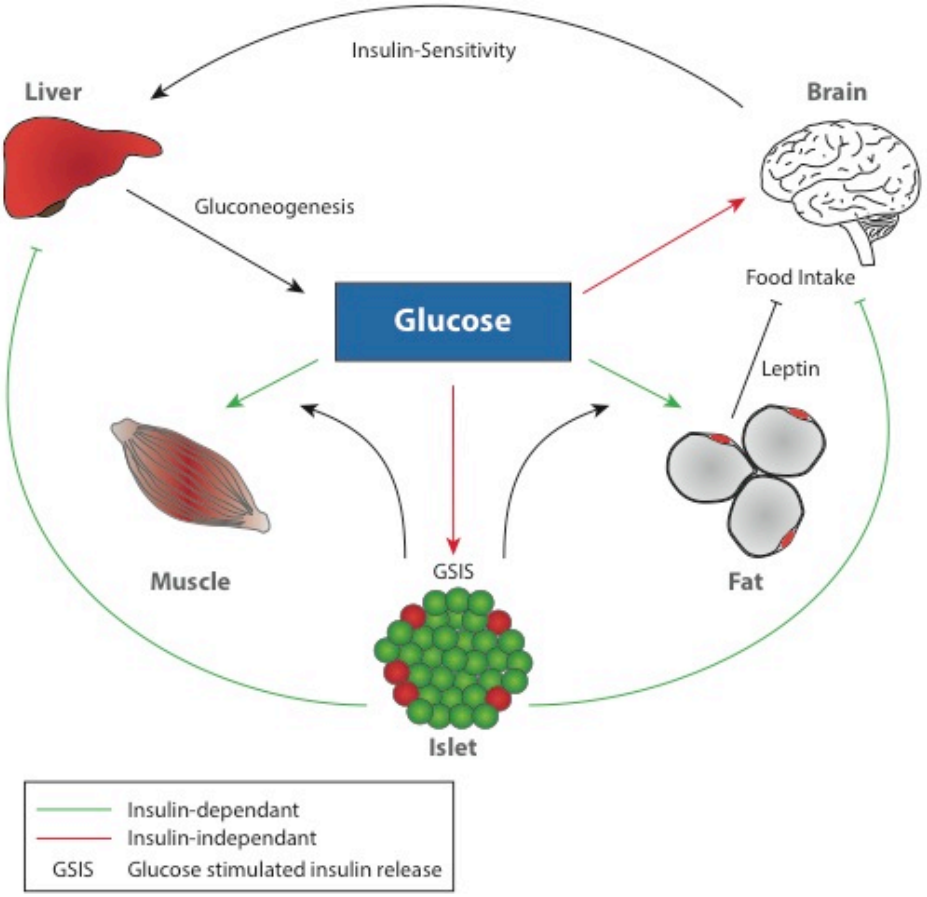

Figure 1: Crosstalk between tissues in the regulation of glucose homeostasis.
An increase in blood glucose levels stimulates the release of insulin from pancreatic beta cells. Insulin promotes the uptake of glucose in muscle and fat tissue, but also suppresses gluconeogenesis in the liver. The brain takes up glucose independent of insulin and regulates insulin sensitivity in the liver. Insulin and fat tissue-derived leptin suppresses food intake that is controlled by the hypothalamic neurons of the brain.

1.1.4 Central regulation of glucose homeostasis

During the mid-19th century, studies by Claude Bernard indicated the importance of brain in the regulation of glucose homeostasis (Bernard, 1854). After the discovery of the pancreatic islet, insulin and insulin responsive tissues including the liver, muscle and fat, the importance of the brain declined further (Schwartz et al., 2013). Instead, the mechanisms underlying T2D were explained on the basis of molecular defects in the release of insulin or its action (Biddinger and Kahn, 2006). However, central mechanisms account for about 50 % of overall glucose disposal (Best et al., 1996). The insulin receptor is also widely expressed throughout the brain with the highest site in the olfactory bulb, followed by the cortex, the hippocampus and the hypothalamus (Kleinridders et al., 2014). The hypothalamus is the best-characterized brain region with respect to glucose metabolism. It consists of first-order neurons expressing both, insulin

as well as leptin receptors (Varela and Horvath, 2012). This region lacks an effective blood brain barrier and allows sensing of changes in hormone and nutrient level in the blood stream (Rodriguez et al., 2010). Pro-opiomelanocortin (POMC) and Agouti-related peptide (AgRP) neurons are the best characterized neuronal populations in the hypothalamus and regulate food intake, insulin sensitivity and energy expenditure (Gao and Horvath, 2007). In contrast, recent evidence suggests the existence of insulin-independent mechanism of glucose homeostasis. For instance, the infusion of leptin in the brain in insulin deficient mice was sufficient to normalize blood glucose level (German et al., 2011; Perry et al., 2014b).

1.1.5 The pancreatic islet

Among the tissues that contribute to the regulation of blood glucose, the pancreas is among the best-characterized organs. The pancreas is a gland consisting of exocrine and endocrine parts. While the exocrine pancreas produces and releases digestive enzymes into the intestine, the endocrine part releases four different hormones into the blood stream. Endocrine cells form clusters within the pancreas, called pancreatic islets. They consist of insulin producing beta, the glucagon producing alpha, the pancreatic polypeptide producing gamma and the somatostatin releasing delta cell types (Edlund, 2002). The beta cell is the most dominant cell type that makes up 65 to 80 % of the islet cell population, followed by alpha (15 to 20 %), gamma (3 to 5 %) and the delta cell types (3 to 10 %). In mice, the pancreas develops around embryonic day E8.5 due to budding of the dorsal and ventral pancreas from the primitive gut epithelium, which is driven by the transcription factor pancreatic and duodenal homeobox gene 1 (*Pdx1)* (Edlund, 2002). Transcriptional programs ultimately drive the differentiation into defined exocrine and endocrine cell types. While the pancreatic transcription factor Ptf1a drives the development of the exocrine tissues, Ngn3 promotes differentiation of the endocrine lineage, followed by further specification of the four different cell types (Jorgensen et al., 2007). The specification of all relevant cell types in the pancreas is complete by day E12.5.

Pancreatic islets are highly vascularized and innervated, which is relevant for their function. Though islets form only 2 % of the total pancreatic volume, they receive 10 % of the total blood flow in the pancreas (Brissova and Powers, 2008). Proper blood flow is crucial, as it enables the cells to quickly respond to the metabolic demand and release hormones and other signaling molecules (Lammert, 2008). The mutual signaling and

functional consequences between blood vessels and beta cells is well characterized. During early development, beta cells release vascular endothelial growth factor (VEGF), which recruits endothelial cells (EC) to the islet. This ensures a dense capillary network surrounding the endocrine cells within the pancreas. Loss of VEGF during development results in decreased vessel density, reduced beta cell mass and impaired glucose tolerance (Reinert et al., 2013). In contrast, loss of VEGF in adult islets has only a marginal effect on vascularization and glucose homeostasis. Besides its role in supply of oxygen and nutrients, ECs form a basement membrane, which is shared with the neighboring beta cell and promotes proliferation and the release of insulin (Eberhard et al., 2010; Eberhard and Lammert, 2009; Lammert et al., 2001; Nikolova et al., 2006). During insulin resistance, the vasculature of pancreatic islets undergoes morphological changes. Here, the *ob/ob* pancreatic islet vasculature adapts due to dilatation, the formation of a thicker basement membrane and an increased parasympathetic innervation (Dai et al., 2013). The innervation of the islet is also crucial for beta cell function, as it regulates vascular scaffolding and might modulate the regulation of hormone release (Reinert et al., 2014). Taken together, proper islet innervation and vascularization is important to islet function and might also contributes to compensatory responses during insulin resistance and beta cell failure.

1.1.5.1 Glucose stimulated Insulin secretion (GSIS)

The main function of the pancreatic beta cell is to act as glucose sensor and release insulin in response to increased plasma glucose levels. The molecular basis and the involved mechanisms are well characterized (Figure 2). In the beta cell, glucose is efficiently taken up via the Glut2 transporter, which is also expressed in the liver and kidneys. Glut2 is unique in its low substrate affinity and allows for a faster transport of glucose (Thorens and Mueckler, 2010). Once glucose enters the beta cell, it is rapidly phosphorylated by Glucokinase (Gck) and is metabolized via the glycolytic and oxidative phosphorylation pathways. The phosphorylation by Gck is the rate-limiting step during glucose stimulated insulin release (Doliba et al., 2012). Glucose metabolism results in an increased ATP/ADP ratio, which closes the ATP sensitive potassium channel (K^+_{ATP}) and generates an action potential (Misler et al., 1992; Misler et al., 1989). The action potentials in beta cells result in extracellular Ca^{2+} influx, which is the ultimate trigger for the fusion of insulin containing vesicles with the cell membrane. The release of insulin is biphasic and can be explained by the existence of different

pools of vesicles. The first phase of insulin release is characterized by a rapid response due to fusion of pre-docked vesicles with the plasma membrane. The amount of insulin during the second phase is slightly reduced, compared to the first phase. Here, insulin secretion reaches a steady state by recruitment of new insulin containing vesicles to the membrane (Michael et al., 2007; Rorsman and Renstrom, 2003).

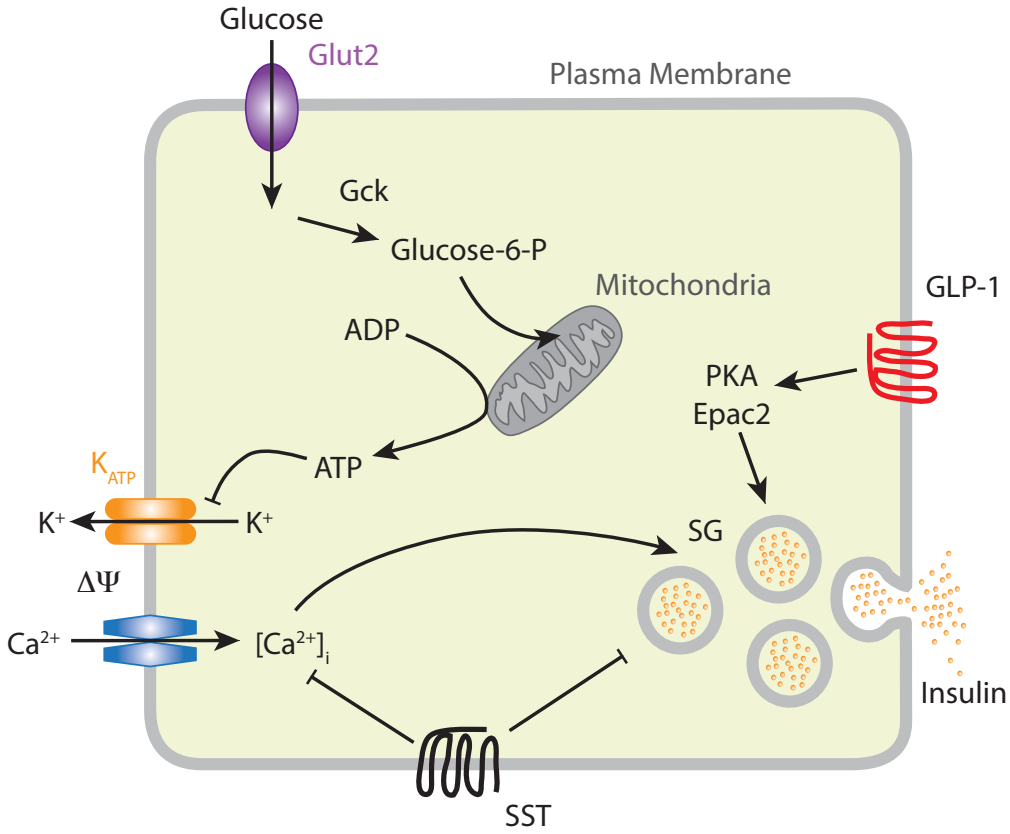

Figure 2: Mechanism of glucose stimulated insulin release.
Glucose is taken up by the Glut2 transporter and rapidly metabolized via glycolytic and oxidative phosphorylation pathways. An increase in cellular ATP levels facilitates closure of an ATP sensitive K^+ channel and subsequently depolarizes the membrane. The following influx of Ca^{2+} promotes the fusion of the insulin containing granules with the cell membrane and hence the release of insulin. The pathway is negatively regulated by Somatostatin (SST) due to reduced action potential firing. GLP-1 enhances the release of insulin by activation of PKA and Epac2, which promotes granule fusion.

Circulating peptides and neurotransmitters can further modify the insulin secretion pathway. Glucagon-like peptide-1 (GLP-1) is released by intestinal L-cells in response to ingestion and promotes insulin secretion by increasing intracellular cAMP concentrations and by enhancing the inhibitory effect on the potassium channel (Gromada et al., 1998). In contrast, the delta cell derived somatostatin inhibits the release of insulin by rapid membrane repolarization and the inhibition of action potential firing (Singh et al., 2007). Stimulating the beta cells to release insulin is one

of the broadly used strategies to treat diabetes. Secretagogues such as Sulphonylureas and Meglitinides both target the regulatory subunit of the K^+_{ATP} channel Sur1 and enhance the release of insulin. Another class of drugs includes the GLP-1 analogues or inhibitors of dipeptidyl peptidase 4 (DPP4), the enzyme that degrades GLP-1 (Vetere et al., 2014).

1.1.5.2 Stress responses of the beta cell and beta cell failure

Insulin is required to maintain plasma glucose levels within the normal range of 4-6 mM. Under healthy conditions, glucose stimulated insulin release is sufficient to counter acute hyperglycemia after food intake and normalize blood glucose levels within a short time frame. However, chronic metabolic stress situations require adaptation of the pancreatic islet and an increased production of insulin in order to maintain blood glucose levels within the physiological range. The islets can respond to such demands by either increasing the amount of secreted insulin (Cavaghan et al., 2000), reducing the threshold for glucose stimulated insulin release or promoting an increase in beta cell mass (Sachdeva and Stoffers, 2009; Sorenson and Brelje, 1997). During insulin resistance, peripheral tissues lose their capability to respond to insulin thus constituting a major driver in the pathogenesis of T2D. However, the maintenance of glucose homeostasis mainly depends on the beta cells to compensate for the increased demand of insulin (Chen et al., 2012). Pregnancy is another metabolic stress condition and is a physiological state of insulin resistance (Rieck and Kaestner, 2010). Recent studies identified several factors derived from different tissues, which circulate in the blood stream and promote beta cell expansion. Glucose and insulin have been shown to promote beta cell proliferation (Otani et al., 2004; Porat et al., 2011). GLP-1 was also shown to promote beta cell replication in a rat model of diabetes (Farilla et al., 2002). The liver-derived betatrophin is another protein hormone that was recently identified to promote beta cell replication. However, its exact biological function and contribution remains controversial (Wang et al., 2013b; Yi et al., 2013). Pituitary derived prolactin and placental lactogen play a special role during pregnancy and have also been shown to promote beta cell hyperplasia during pregnancy (Sachdeva and Stoffers, 2009).

In addition to insulin resistance, beta cell failure is considered to be another key feature during the pathogenesis of T2D. Beta cell failure is defined as a loss of functional beta cell mass and is, at least in final stages of the disease, characterized by beta cell death

(Rhodes, 2005). However, recent advances revealed that beta cell failure is a complex phenomenon caused by various factors including ER stress, amyloid stress, inflammation, and oxidative stress (Halban et al., 2014). The majority of identified genomic risk loci are linked to defects in insulin secretion, altering granule docking and Ca^{2+} sensitivity (Rosengren et al., 2012). Recently, the group of Domenico Accili discovered another mechanism of beta cell failure (Talchai et al., 2012). They could show that upon various forms of metabolic stress, beta cell de-differentiate into a progenitor-like state, which does not express insulin. Occasionally, these cells differentiate into glucagon-expressing cells, further contributing to hyperglycemia. These findings facilitate a novel treatment approach, which aims to re-differentiate these progenitor-like cells and restore functional beta cell mass.

Stress responses are accompanied by alterations in gene expression programs and involve transcriptional as well as post-transcriptional mechanisms in order to maintain the physiologic state (Kultz, 2005). This includes the microRNA pathway that has been shown to be a potent posttranscriptional regulator of gene expression.

1.2 The microRNA pathway

1.2.1 The biogenesis and function of microRNAs

The last decades shed light on the mechanism of glucose homeostasis and resulted in the discovery of contributing gene families, including transcription factors, ion channels, trafficking molecules and enzymes of the metabolic pathways. Studying the timing of larval development in the model organism *Caenorhabditis elegans* led to the discovery of the very first microRNAs (miRNA) *lin-4* and *let-7* (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). MiRNAs form a large family of ~22 nucleotide long RNAs, which negatively regulate gene expression by binding to defined complementary sites on mRNAs. Computational prediction suggests that more than half of the protein-coding genes carry miRNA target sites (Friedman et al., 2009). To date more than 2500 human miRNAs have been annotated in the miRBase database (Kozomara and Griffiths-Jones, 2014). MiRNAs are highly conserved and expressed in more than 200 species, including mammals, worms, flies, plants and viruses (Lee et al., 2007). Since their identification in *Caenorhabditis elegans*, the pathway of miRNA processing and incorporation in functional proteins complexes has been well characterized comparably with mRNAs (Figure 3). MiRNAs are transcribed by RNA-

polymerase II and are capped and polyadenylated (Cai et al., 2004; Lee et al., 2004). The transcribed primary-miRNA (pri-miRNA) forms a stem-loop structure, which is recognized and cleaved by a protein complex formed by Drosha and Dgcr8 (Denli et al., 2004; Gregory et al., 2004). This processing step generates precursor miRNAs (pre-miRNAs), which forms ~60 nucleotide long stem-loop structure. Alternatively, pre-miRNAs can also be generated from introns of protein-coding genes during the process of splicing (Kim and Kim, 2007). Pre-miRNAs are subsequently transported by Exportin-5 from the nucleus to the cytoplasm (Yi et al., 2003). In the cytoplasm, pre-miRNAs are further processed by the RNase Type III enzyme Dicer, which cleaves the loop structure giving rise to ~22 nucleotide miRNA-miRNA*-duplex (Hutvagner et al., 2001). This duplex is then transferred to a member of the Argonaute family (Ago), a key component of the RNA-induced-silencing-complex (RISC). Here, the RNA duplex is separated and the miRNA* strand is degraded, resulting in functional RISC complexes (Czech and Hannon, 2011). The miRNA guides the RISC complex to its target mRNAs and suppresses gene expression. However, the interaction between the miRNA and the target mRNA is based on imperfect base pairing (Bartel, 2009). The principles for the interaction have been established by computational as well as experimental approaches (Brennecke et al., 2005; Brodersen and Voinnet, 2009; Didiano and Hobert, 2006; Watanabe et al., 2007). A continuous and perfect Watson-Crick base pairing between nucleotides 2-8 of the 5' -end of the miRNA and its target mRNA characterizes canonical target sites and is often termed "seed-sequence". Additionally, partial interactions between the 3'-end of the miRNA and its target can further stabilize the interaction and also compensate for mismatches within the seed-sequence (Bartel, 2009). There are very few examples in mammals with a high degree of miRNA-target complementarity. For example, miR-196 targets *Hox* genes with almost perfect complementarity (Yekta et al., 2004). A high degree of miRNA-mRNA complementary is more common in plants and is a rather rare event in mammals (Ameres and Zamore, 2013). The majority of predicted and validated binding sites of miRNA are located within the 3' UTR. However, there are also a few examples, where miRNAs can bind within the coding region or the 5' UTR (Orom et al., 2008; Rigoutsos, 2009).

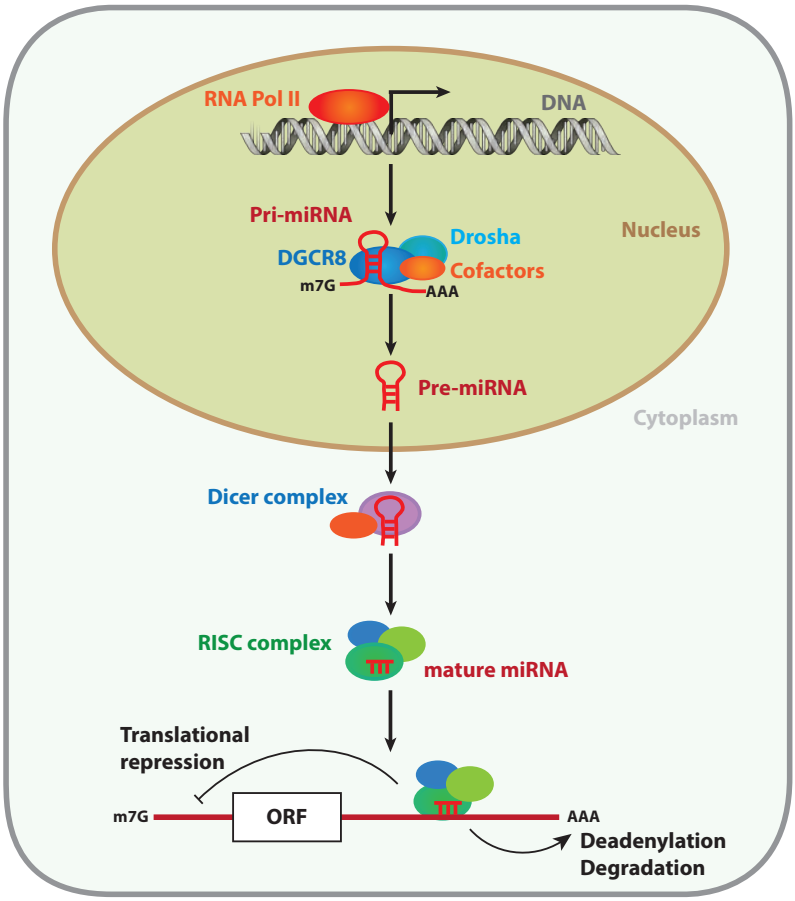

Figure 3: The biogenesis and function of miRNAs.
MiRNAs are transcribed by RNA Polymerase II in the nucleus and gives rise to the Pri-miRNA, which is processed by adding the 5'-cap and a poly(A) tail. The Pri-miRNA forms a stem loop structure that is recognized and cleaved a protein complex formed by DGCR8 and Drosha. The resulting Pr-miRNA is subsequently transported to the cytoplasm, where it undergoes another cleavage by the Dicer complex. The resulting mature miRNA is incorporated into the RISC complex and guides it to its target mRNAs. The miRNA-RISC complex binds to 3'UTRs and negatively suppresses expression by translational repression or deadenylation and degradation of the mRNA.

MiRNAs negatively regulate the expression of their target mRNA on the post-transcriptional level. This can be achieved by either inhibition of translation or destabilization of the mRNA transcript, which results in its degradation (Guo et al., 2010; Selbach et al., 2008). In mammals 4 different members of the Ago family exist, but only Ago2 features catalytic endonucleolytic activity and allows cleavage of target mRNA sequences (Meister et al., 2004). In addition to the cleavage by Ago2, target mRNAs can be destabilized by the RISC, also resulting in their degradation. This is accomplished by the recruitment of additional factors to the RISC complex, such as the CCR4-NOT deadenylation complex and the poly(A)-binding protein (PABP)

(Chekulaeva et al., 2011). This also destabilizes the mRNA and recruits the decapping protein Dcp2 to further facilitate the degradation of the mRNA (Behm-Ansmant et al., 2006). Alternatively, miRNAs can also block translation of the mRNA by the recruitment of proteins to the RISC that affect various steps of translation. This includes the initiation of translation by interfering with the assembly of the 40S and 60S ribosomal subunits, the elongation or the degradation of the nascent polypeptide (Fabian et al., 2010). Moreover, GW182, another component of the RISC complex, interferes with the circularization of the transcript and reduces efficient translation (Tritschler et al., 2010).

The subcellular localization is important for the regulation of the miRNA pathway because it unites the functional components of the machinery (Krol et al., 2010). Processing bodies and stress granules have been found to be important for miRNA-mediated repression of target genes. Upon stress, complexes formed by mRNAs and the RISC complexes accumulate in cellular foci, where they are either stored or degraded (Franks and Lykke-Andersen, 2008; Parker and Sheth, 2007). Stress granules represent another subcellular structure and share features with processing bodies. They are formed upon stress and translational inhibition and are also important for the regulation of the miRNA pathway (Anderson and Kedersha, 2008).

1.2.2 Argonaute proteins as mediators of microRNA function

Members of the Ago family proteins are key components of small-RNA mediated gene silencing. They are highly conserved and are expressed in all eukaryotes, with the exception of yeast (Meister, 2013). Loss of Ago2 in mice results in embryonic lethality due to developmental abnormalities including a defect in the closure of the neural tube, mispatterning of the brain and cardiac failure (Liu et al., 2004). Additional studies have addressed the role of Ago2 in various tissues and revealed its function to be involved in cocaine addiction, skin development, hematopoiesis, beta cell function and T-cell activation (Bronevetsky et al., 2013; O'Carroll et al., 2007; Schaefer et al., 2010; Tattikota et al., 2014; Wang et al., 2012). In contrast to other family members of the Argonaute family of proteins, Ago2 is unique in possessing catalytic activity that enables cleavage of perfectly complement target mRNAs (Hauptmann et al., 2013; Meister et al., 2004). In addition, this activity is crucial for the processing and maturation of miR-451, the only known example (Cheloufi et al., 2010; Yang et al., 2010). All Ago members share distinct structural features and feature the following

domains: the amino-terminal (N) domain, the PAZ (Piwi-Argonaute-Zwille) domain, the MID (middle) domain and the Piwi domain (Hock and Meister, 2008). The crystal structure of Ago2 was recently deciphered, revealing a bi-lobed structure (Schirle and MacRae, 2012). The two lobes are formed by N and PAZ as well as MID and PIWI domain respectively and are connected by the L2 linker. Both, the PAZ and the MID domains mediate the RNA interaction. In addition to the RNA-binding, Ago proteins also form a platform to bind various factors, which mediate translational suppression or degradation (Czech and Hannon, 2011). The members of the Ago family are an important component of the miRNA pathway and undergo critical regulation. Ago2 is regulated by *miR-184* during inflammation and insulin resistance (Roberts et al., 2013; Tattikota et al., 2014). Furthermore, Ago function is regulated by posttranslational mechanisms. Ago can be hydroxylated which affects protein stability (Qi et al., 2008). Additionally, Ago2 can by modified by poly(ADP-ribose) which is crucial for the assembly of cytoplasmic stress granules and miRNA activity (Leung et al., 2011). Protein phosphorylation is one of most prominent posttranslational modifications, regulating activity, stability and localization of proteins. Importantly, phosphorylations are connected to changes in the environment and are implicated in stress responses. Ago proteins are also phosphorylated at various residues. Phosphorylation of Ago2 at Serine S387 facilitates its localization to processing bodies (Zeng et al., 2008). Phosphorylation of tyrosine Y529 have been implicated in the regulation small RNA binding (Rudel et al., 2011). Lastly, upon hypoxic conditions Ago2 is phosphorylated at tyrosine Y393 and is associated with reduced binding to Dicer and down regulation of a subset of miRNAs (Shen et al., 2013). The phosphorylation of tyrosine Y393 has also been shown to be important during oncogenic Ras-induced senescence (Yang et al., 2014). Here, the oncogenic activation results in inactivation of the phosphatase PTP1B, resulting in increased phosphorylation of Ago2 and the induction of cell cycle arrest and cellular senescence. This further underlines the importance of the miRNA pathway as a mediator of stress responses.

In addition to its function in the posttranscriptional regulation of gene expression, Ago proteins have been found to be localized within the nucleus where it potentially regulates chromatin modifications, alternative splicing and DNA double-strand break repair (Meister, 2013).

1.2.3 The functional role of the microRNA pathway

Many insights into the functional role of miRNAs have been recently made, improving our understanding of how these small RNAs integrate into the already complex landscape of regulating gene expression (Rathjen et al., 2014). Loss of function studies of important components of the miRNA pathway such as Dicer, Drosha and Ago2 highlighted their importance during development (Bernstein et al., 2003; Fukuda et al., 2007; Liu et al., 2004). Genetic deletion of either of these genes resulted in embryonic lethality. Moreover, specific miRNA knockout mice also indicate a functional relevance during development. Loss of *miR-1* and the *miR-17-92* cluster results in embryonic lethality (Ventura et al., 2008; Zhao et al., 2007).

Remarkably, the majority of published miRNA-knockout models show only minor phenotypes, suggesting a role in cellular stress responses (Bushati and Cohen, 2007; Miska et al., 2007; Park et al., 2010). Dramatic phenotypes are usually more common once the miRNA loss of function model experiences various forms of stress (Xin et al., 2009). Cellular stress is defined as a deviation from steady state physiology and is caused by changes in the environment. In order to adapt to and counter this situation cells need to modulate signaling and metabolic pathways in order to reestablish cellular homeostasis (Leung and Sharp, 2010). This requires changes in gene expression and is partially mediated by the miRNA pathway. There are different models established, which mediate these adaptations, including changes in the expression level of miRNA, modifications of the activity of the RISC complex, modulation of the availability of the seed sequences within in targets due to alterative splicing (Leung and Sharp, 2010). In summary, the miRNA pathway is an important regulator of cellular stress responses and crucial to maintain normal physiology.

1.2.4 The microRNA pathway and metabolic diseases

While many functions of the miRNA pathway has been linked to development and the maintenance of cellular function, it also has been implicated in the pathogenesis of various diseases, including cancer, cardiovascular, metabolic and neurologic disorders (Chang and Mendell, 2007; Hata, 2013; Maciotta et al., 2013; Rottiers and Naar, 2012). For instance, LIN28 is negative regulator of the *let-7* family of miRNAs and is up regulated during tumor genesis (Viswanathan et al., 2009). Here, increased expression of LIN28 resulted in suppression of *let-7* miRNAs and de-repression of its targets *c-*

Myc and *K-Ras*, two driver of tumor genesis. Moreover, the LIN28/*let-7* axis also plays an important role during glucose homeostasis. Increased expression of LIN28 resulted in improved insulin sensitivity and is caused by de-repression of multiple *let-7* targets within the mTOR pathway. Interestingly, several target genes of *let-7* have also been identified in GWAS studies and are related to T2D (Zhu et al., 2011). The miRNA pathway has been also linked to aging and the regulation of age-related stress responses. Mori and colleagues could show that Dicer is down regulated in adipocytes during aging and is linked to the reduction in miRNA expression. In contrast administration of a caloric diet can restore dicer expression and increases life span (Mori et al., 2012). *MiR-103* and *miR-107* are among the most-up regulated miRNA in white fat and livers of genetically or diet-induced obese mice (Trajkovski et al., 2011). These miRNA have been shown to target *Caveolin1* and are implicated in the regulation of insulin sensitivity. Restoring *miR-103* and *miR-107* expression levels greatly improved insulin sensitivity and glucose homeostasis. Another example is *miR-133* that regulates heat production in brown adipose tissue (BAT) in mice. Upon cold challenge, *miR-133* is down regulated and mediates expression of *Prdm16* that regulates Ucp1 expression in BAT (Trajkovski et al., 2012). Ucp1 uncouples the respiratory chain and generates heat (Kajimura et al., 2010). Taken together the misexpression of miRNAs or components of the pathway have been shown to contribute to the pathogenesis of multiple diseases and constitutes an interesting therapeutic target.

1.2.5 Beta cell function is regulated by the microRNA pathway

Several studies have addressed the role of the miRNA pathway in the pancreatic beta cell, both during development as well as a regulator of cellular function. The development of the pancreas in mice starts at day E9.5 (Edlund, 2002). Several studies have addressed the role of the miRNA pathway in the developing pancreas by genetically ablating dicer (Lynn et al., 2007; Mandelbaum et al., 2012). Consistently, both studies show that loss of Dicer in the pancreas or beta cells resulted in gross developmental defects of the tissue and reduced beta cell mass. Furthermore, mice develop hyperglycemia and show impaired glucose tolerance, suggesting that the miRNA pathway is crucial for the development of the endocrine pancreas. In order to further address the role of Dicer in fully developed pancreatic islets, Dicer was conditionally deleted in differentiated beta cells (Melkman-Zehavi et al., 2011). Loss of Dicer resulted in hyperglycemia and loss of insulin expression, though islet architecture

remained normal. This data suggest that the miRNA pathway is involved in the development, maintenance of beta cell identity and crucial for the function in order to regulate blood glucose level (Tattikota and Poy, 2011).

In addition to total loss of the miRNA pathway, several studies addressed individual miRNAs to get more insight in the biological function of certain miRNAs. The very first report of the function of miRNAs in beta cells came from the lab of Markus Stoffel. The group identified *miR-375* among the most abundant miRNAs in the pancreatic beta cell line MIN6 (Poy et al., 2004). Secretion studies in MIN6 cells and isolated mouse islets revealed that *miR-375* is a negative regulator of insulin secretion by targeting myotrophin (Mtpn). Furthermore, genetic ablation of *miR-375* demonstrated that this miRNA is essential for maintaining beta cell mass in mice (Poy et al., 2009). Moreover, loss of *miR-375* during resulted in loss of compensatory beta cell expansion during insulin resistance in leptin deficient *ob/ob* mice. Several novel targets, including *Elavl4*, *RasD1* and *Cadm1*, have been identified. However, their biological function in the pancreatic beta cell still remains elusive. The role of *miR-375* in the regulation of beta cell mass is also conserved in zebra fish (Kloosterman et al., 2007). The intrauterine environment is also considered to be risk factor for the development of T2D (Chen et al., 2012). Dumortier and colleagues could show that low protein diet during pregnancy resulted in significant higher level of *miR-375* in the offspring. The authors link this observation to glucose intolerance in the offspring by targeting *Mtpn* and *Pdk1*, suppressing glucose stimulated release of insulin and proliferation (Dumortier et al., 2014). *MiR-7* is another abundant miRNA in the endocrine pancreas and is known to regulate the differentiation of endocrine cells by targeting the transcription factor Pax6 (Kredo-Russo et al., 2012). Furthermore, *miR-7* is involved in the regulation of beta cell proliferation by targeting different components of the mTOR pathway (Wang et al., 2013a). In contrast, a study by the Stoffel group did not observe any effect of *miR-7* on proliferation or the rate of apoptosis. Moreover, they could show a role of *miR-7* in the regulation of insulin release and the adaptation of the pancreatic beta cell during insulin resistance (Latreille et al., 2014).

1.2.6 *miR-375* targets the cell adhesion molecule *Cadm1*

Several computational algorithms predict about 230 conserved targets for *miR-375* (www.targetscan.org; pictar.mdc-berlin.de). One of the predicted genes is the cell adhesion molecule 1 (*Cadm1*) that has one conserved *miR-375* binding site in its 3'UTR

(Poy et al., 2009). Cadm1 is a member of the immunoglobulin superfamily and consists of three extracellular immunoglobulin domains, a trans-membrane domain and a short cytoplasmic domain (Murakami, 2005) (Figure 4). It mediates cell-cell interaction via homo- and heteromeric interaction with itself or its family member Cadm2 (Fogel et al., 2007). Cadm1 is expressed in all endocrine cell types in the pancreatic islet and potentially mediates the interaction with neighboring endocrine cells, as well as neurons and blood vessels (Ito et al., 2012; Koma et al., 2008; Tatsumi et al., 2012). Originally, Cadm1 was identified as a tumor suppressor in lung cancer and its promoter is frequently methylated (Kuramochi et al., 2001; Murakami, 2002). Mao and colleagues could show that tumor suppressive function depends on the cytoplasmic domain (Mao et al., 2003). Loss of function studies in mice could also show that Cadm1 is involved in spermatogenesis (van der Weyden et al., 2006).

Cadm1 is also expressed at high levels in the brain, where it mediates the interaction between pre- and post-synaptic neurons (Biederer et al., 2002). Interestingly, expression of Cadm1 and a glutamate transporter was sufficient to form functional contacts between non-neuronal cells. Moreover, loss of function studies of Cadm1 in mice resulted in a decrease number of excitatory synapses, while overexpression showed increased numbers (Robbins et al., 2010). Trans synaptic adhesion molecules are thought to organize the synapse by establishing protein complexes that stabilize the interaction, but also play an important role for the synaptic transmission (Fogel et al., 2007). Moreover, the extracellular domain of Cadm1 is glycosylated and is crucial for its binding affinity. Lastly, Cadm1 is involved in the formation of neuronal networks, synaptic plasticity and learning. Loss of Cadm1 results in reduced spatial learning (Robbins et al., 2010). While previous studies of Cadm1 focused on its role as a tumor suppressor, spermatogenesis and its function during the learning processes, nothing is known about the functional role of Cadm1 in the regulation of glucose homeostasis.

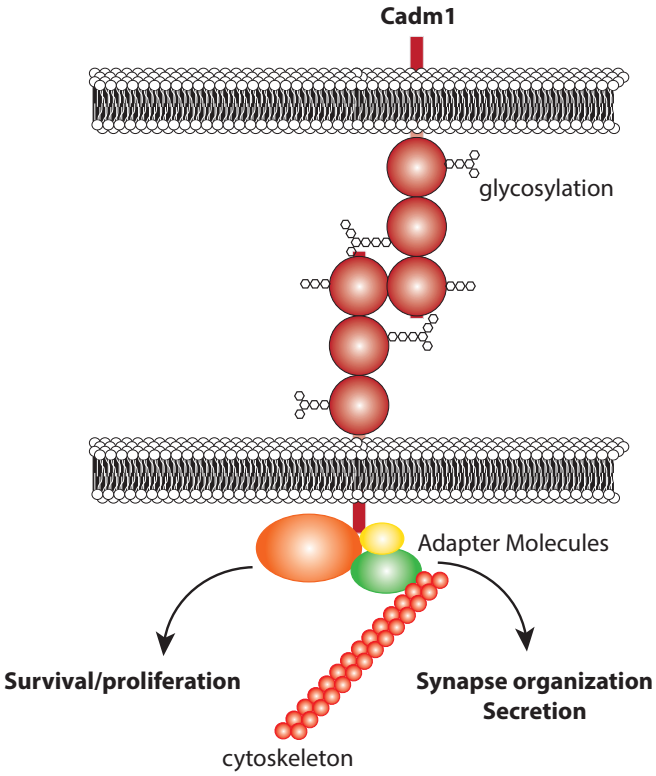

Figure 4: Cadm1 mediates cell-cell-contacts and regulates cellular function. Cadm1 is a glycosylated cell adhesion molecule that regulates the interaction between two neighboring cells. It is known to interact with multiple cytosolic proteins and the cytoskeleton and is involved in the regulation of survival and proliferation, the organization of synapses and the release of insulin.

2 Aim of the study

The miRNA pathway has been established as an important regulator of gene expression and contributes to the maintenance of cellular function, both during steady state as well as stress conditions. While many studies addressed the function of single miRNAs in the pancreatic beta cell, little is known about the contribution of Argonaute2 (Ago2), an important mediator of miRNA function. Hence, the aim of this study is to characterize the functional role of Ago2 in the pancreatic beta cell and understand how it exerts its function in terms of the regulation of beta cell proliferation, the release of proteins from the beta cell and stress responses. Moreover, this study tries to understand what are important target genes of the miRNA pathway, particularly *miR-375* that is among the most abundant miRNAs in the beta cell. Lastly, this thesis aims to understand how important targets mediate the function of the miRNA pathway and how do they globally contribute to the regulation of glucose and energy homeostasis, both during normal as well as disease conditions. Here, we will focus on the functional characterization of the Cell adhesion molecule 1 (Cadm1), which is a conserved target gene of *miR-375* in the pancreatic beta cell, but is also expressed abundantly in the brain. This will shed light on the functional role of the miRNA pathway and further aims to understand how different tissues orchestrate the regulation of energy homeostasis.

3 Materials and Methods

3.1 Materials

3.1.1 Devices

Table 1: Devices

Device	Supplier
Amaxa Nucleofector II	Lonza
WIZARD2® Automatic Gamma Counter	PerkinElmer
SP5 Confocal Microscope	Leica
Contour Glucometer	Bayer
Fusion FX Chemiluminescence Scanner	PEQLAB
StepOne Real-Time PCR System	Applied Biosystems
Infinite 200 microplate reader	Tecan
NanoDrop ND-1000	PEQLAB
TSE Phenomaster	TSE Systems

3.1.2 Chemicals

If not stated differently, all chemicals have been obtained from Carl Roth GmbH & Co. KG, Sigma Aldrich or Life Technologies.

Table 2: Chemicals

Chemical	Supplier
Histopaque®-1119	Sigma Aldrich
Doxycycline	AppliChem
Human Insulin	PAN Biotech

3.1.3 Kits

Table 3: Kits

Kit	Supplier
SuperSignal West Femto Chemiluminescent Substrate	Thermo Fisher Scientific
RNeasy Mini Kit	QIAGEN
Rat Insulin RIA Kit	Millipore
Glucagon RIA Kit	Millipore
Amaxa Nucleofector Kit V	Lonza
MicroRNA TaqMan® Assays	Life Technologies
In Situ Cell Death Detection Kit	Roche
TaqMan® MicroRNA Reverse Transcription Kit	Life Technologies
FastStart SYBR Green Master	Roche
Plasmid Maxiprep PureLink HiPure	Life Technologies
QIAquick Gel Extraction Kit	QIAGEN
TriZol®	Life Technologies
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher Scientific

3.1.4 Enzymes

Table 4: Enzymes

Enzyme	Supplier
Restriction Endonucleases	New England Biolabs
Collagenase (C9407)	Sigma Aldrich
Phusion® High-Fidelity DNA Polymerase	New England Biolabs
Taq DNA Polymerase	Life Technologies

3.1.5 Antibodies

Table 5: Primary Antibody for Western Blotting

Antibody	Species	Dilution	Supplier
Ago2	Rabbit	1:1000	Cell Signaling
γ-Tubulin	Mouse	1:1000	Sigma Aldrich
α-Actin	Rabbit	1:1000	Sigma Aldrich
HuD	Rabbit	1:500	Santa Cruz
Cadm1	Chicken	1:1000	MBL
Gphn	Rabbit	1:1000	BD Bioscience
Ywhaz	Rabbit	1:1000	Abcam
Rasd1	Rabbit	1:1000	Millipore
Ago1	Rabbit	1:1000	MBL
Akt	Rabbit	1:1000	Cell Signaling
Phospho-Akt (Ser473)	Rabbit	1:1000	Cell Signaling
Pten	Rabbit	1:1000	Cell Signaling

Table 6: Primary Antibody for Immunofluorescence

Antibody	Species	Dilution	Supplier
Insulin	Guinea Pig	1:500	Millipore
Glucagon	Rabbit	1:500	Millipore
CD31	Rat	1:50	BD Bioscience
BrdU	Rat	1:200	Abcam

Table 7: Secondary Antibody for Western Blotting

Antibody	Species	Dilution	Supplier
Anti-Mouse-HRP	Goat	1:10000	Calbiochem
Anti-Rabbit-HRP	Goat	1:10000	Calbiochem
Anti-Rat-HRP	Goat	1:10000	Calbiochem
Anti-Chicken-HRP	Goat	1:10000	Santa Cruz

Table 8: Secondary Antibody for Immunofluorescence

Antibody	Species	Dilution	Supplier
Anti-Guinea Pig-Alexa-488	Goat	1:500	Life Technologies
Anti-Rat-Alexa-555	Goat	1:500	Life Technologies
Anti-Rabbit-Alexa-555	Goat	1:500	Life Technologies

3.1.6 Cell lines

All cell culture experiments were performed in the murine pancreatic beta cell line MIN6 (Miyazaki et al., 1990).

3.1.7 Bacterial strains

All cloning procedures were performed in DH5α™ *E. coli* strain, which were obtained from life technologies.

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Culture of pancreatic beta cell line MIN6

The murine insulinoma cell line MIN6 was cultured in DMEM (GIBCO) containing 4.5g/l glucose supplemented with 15 % v/v heat-inactivated FCS, 50 μM β-mercaptoethanol, 1 % penicillin/streptomycin in a humidified incubator at 37°C and 5 % CO_2. The media was changed every three days and cells were passaged after reaching a confluency of about 80 %. Therefore cells were washed twice with 1xPBS and trypsinized with 0.05 % Trypsin (GIBCO), centrifuged for 5 min at 200 g and the cell pellet re-suspended in media.

3.2.1.2 Transfection

For loss and gain of function studies, MIN6 cells were transfected using the Amaxa Nucleofector II (Lonza) according to the manufacturer's instructions and using the transfection kit V. In brief, cells were trypsinyzed as previously described. Two million cells were centrifuged at 90 g for 10 min at room temperature. The pellet was re-suspended in 100 μl transfection solution and supplemented with either 200 pmol siRNA or 4 μg plasmid DNA. As a control a pool of non-targeting siRNA or a plasmid containing an EGFP expression cassette was used. The cells were transferred to a cuvette and electroporated, using program G16. Afterwards, cells were re-suspended in media and seeded in 6- or 24-well plates. Cells were analyzed 48 h post-transfection.

3.2.1.3 Insulin secretion assay

To measure the release of insulin, MIN6 cells were seeded following the transfection in 24-well plates. For secretion, cells were washed once with secretion buffer and afterwards primed for 30 min in secretion buffer containing 5.5 mM glucose. Subsequently buffer was replaced by 1 ml of secretion buffer containing either 2.8 (low) or 25 mM (high) glucose and incubated at 37°C for 1 h. Afterwards the supernatant was collected, centrifuged 5 min at 200 g to remove dead cells and insulin was measured using a RIA kit (Millipore). The release of insulin was normalized to insulin content of MIN6 cells. Therefore, cells were lysed in 1 ml of acid ethanol (1.5 % HCl, in 70 % ethanol) for 10 min at 4°C, centrifuged at 14.000 g for 10 min at 4°C and insulin was measured in the supernatant using a RIA kit (Millipore).

Secretion buffer:

0.54 mM $CaCl_2$,
4.74 mM KCl,
1.19 mM KH_2PO_4,
1.19 mM $MgCl_2$
119 mM NaCl,
25 mM $NaHCO_3$
10 mM HEPES,
0.5 % BSA
in 1000 ml H_2O, pH 7.4, sterile filtered

3.2.1.4 SILAC Labeling and LC-MS/MS

Sudhir Gopal Tattikota and Matthias Sury performed the SILAC and mass spectrometry experiments. For SILAC labeling, MIN6 cells were grown in high glucose DMEM (PAA Laboratories GmbH, Pasching, Austria) supplemented with 15% dialyzed FCS (Sigma-Aldrich), 4 mm glutamine (PAA Laboratories GmbH, Pasching, Austria), 100 units/ml penicillin, 50 µm β-mercaptoethanol, 100 µg/ml streptomycin (Life Technologies), 48 µg/ml Lys-$C_6H_{14}N_2O_2$ (Lys-0) and 28 µg/ml Arg-$C_6H_{14}N_4O_2$ (Arg-0) (Sigma-Aldrich), or 48 µg/ml Lys-$^{13}C_6$ $H_{14}{}^{15}N_2O_2$ (Lys-8) and 28 µg/ml Arg-$^{13}C_6H_{14}{}^{15}N_4O_2$ (Arg-10) (Sigma Isotec). Eight passages in "heavy DMEM " were conducted to achieve a high degree of incorporation of the heavy isotopes into proteins (95.12 ± 4.3% labeling efficiency). For secretion experiments, SILAC-labeled MIN6 cells were washed six times before incubating for 1 h in modified Krebs-Ringer buffer containing either low (2.8 mm) or high (25 mm) glucose, as described previously (Poy

et al., 2004). Supernatants of light and heavy MIN6 cell cultures were combined and concentrated with Amicon Ultra-15 centrifugal filter units with a 3-kDa cutoff (EMD Millipore Corp.). Proteins were precipitated and dissolved in 6 m urea, 2 m thiourea. Proteins were reduced and alkylated. Lysyl endopeptidase (LysC) (Wako, Osaka, Japan) and trypsin (Promega, Madison, WI) were used for in-solution digestion. Stop and go extraction (STAGE) tips containing C_{18} empore disks (3M, Minneapolis, MN) were used to purify and store peptide extract (Rappsilber et al., 2003). LC-MS/MS analysis was done as described previously (Tattikota et al., 2013).

3.2.2 Molecular Biology

3.2.2.1 RNA extraction

Total RNA was extracted as described in the manufactures' instructions. In brief, isolated islets or cells were homogenized in TriZol (Life Technologies) reagent and incubated for 10 min at room temperature. Afterwards 200 µl chloroform per ml TriZol was added and samples were vortexed, followed by centrifugation at 12.000g and 4 °C. The RNA containing upper aqueous phase was transferred to a new tube and RNA was precipitated by adding 500 µl of 2-propanol overnight at -20 °C. The precipitated RNA was pelleted at 12.000 g at 4 °C and washed once with 1 ml of 75 % ethanol. Afterwards, RNA was pelleted again for 5 min at 7.500 g, dried at room temperature and resuspended in RNase free water. RNA concentrations were measured using the NanoDrop photometer.

3.2.2.2 cDNA synthesis

cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific. Therfore 500 to 1000 ng of total RNA were reversed transcribed using a pool of random hexamers and oligo-dT primer for 60 min at 42 °C in the presence of dNTPs, RNase inhibitor and RervtAid reverse transcriptase. Afterwards the reaction was terminated for 5 min at 70 °C. cDNA was diluted 1:5 in nuclease free water and stored at -20 °C.

3.2.2.3 Quantitative realtime PCR for mRNA transcripts

Quantitative realtime PCR was used to measure differences in RNA expression, using the FastStart SYBR green PCR kit from Roche. In brief, a master mix was prepared for 10 µl reactions, containing 2x FastStart SYBR Green Master Mix and 600 nM gene

specific primer mix. The PCR reaction was carried out in a 96-well format, combining 9 µl of the master mix and 1 µl cDNA template and using the Applied Biosystems StepOne Real-Time PCR System. Relative expression levels were calculated using the $2^{(-\text{Delta Delta CT})}$ Method (Livak and Schmittgen, 2001).

Table 9: Primer Sequences for real time PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Cadm1	CTCGAGCAGGTGAAGAGGGGACC ATTGGG	GATGAAGTACTCTTTCTTTTCTTCG GAGT
Ago2	GCACTTACCATCCATGAGGTAC	AAAGAGAAGGTCGGACGGACTGAT
36B4	TGCCAGGACGCGCTTGT	GGCCCTGCACTCTCGCTTTC
Gck	CGTTGACTCTGGTAGAGCAGATC	GAGAAAGTCTCCAACTTCTGAGC
Elavl4	CAGGGATGCTAACCTGTATG	CTTTGATGGCTTCTTCTGCC
Ins1	TCTTCTACACACCCATGTCCC	GGTGCAGCACTGATCCAC
Gphn	TGGTCCAGGGGATCGTTTCAT	TTGTAACCCGCATCACTTGTC
Mtpn	CCCTGAAAAACGGAGACTTGG	GAAACATGACCCTCATAGACAGC
Pdcd8	TCCTCCGGCCCATGATTAAAG	CTGTCTGGCTGCCCATTCT
Ywhaz	AGAAGATCGAGACGGAGCTG	TTGTCATCACCAGCAGCAAC
Clock	AGATCAGTTCAATGTCCTCA	TGTCGAATCTCACTAGCATC
Rgs16	GAGAAGCTTCTCAGAAGATGTACT	GTCTCGTGATCTATGTTCACCTC
Qk	TGTTGGGAGAATCCTTGGAC	CTTCAACCGCTCTCTTCAGC
Rasd1	GATGTGCCCAAGCGACTCT	TGAGGAAGCGCGACACAAT

3.2.2.4 Quantitative realtime PCR for microRNAs

MiRNAs were quantified using TaqMan miRNA Assays from Life Technologies according to the manufactures protocol. For cDNA synthesis 10 ng of total RNA were reverse-transcribed using a miRNA specific looped primer in a 15 µl reaction and the miRNA Reverse Transcription kit from Applied Biosystems. Subsequently, 1 µl of cDNA was used to run the qPCR reaction in a total volume of 15 µl and using miRNA-specific Primer. U6 small nuclear RNA was used as a normalizer. The qPCR reaction was performed in 96-well plates and PCR reaction was monitored using the StepOne Real-Time PCR System from Applied Biosystems.

3.2.3 Animals

3.2.3.1 Animal care

Mice were maintained on a 12-hour light/dark cycle with ad libitum access to regular chow food, high fat diet (containing 60% kcal fat, cat. no. E15741-347, ssniff Spezialdiäten GmbH), or ketogenic diet (cat. No. E15149-30, ssniff Spezialdiäten GmbH) in accordance to requirements established by Landesamt für Gesundheit und Soziales (LAGeSo). All experimental procedures were approved under protocols G 0357/10, O 0405/09, and T 0436/08. miR-375KO animals were kindly provided by Markus Stoffel (Poy et al., 2009). $Ago2^{flox/flox}$ mutant mice were kindly provided by Donal O'Carroll (O'Carroll et al., 2007) and crossed to Ins-Cre mice, provided by Thomas Jentsch (Herrera, 2000). RIP-Tag mice were purchased from Jackson Labs and crossed to the miR-375KO or βAgo2KO mice. Cadm1KO and $Cadm1^{flox/flox}$ mutant mice (van der Weyden et al., 2006) were characterized after backcrossing for four generations to C57Bl6 (Charles River). *Vglut2*-Cre and *Vgat*-Cre expressing mice were purchased (Jackson Labs) and directly crossed to $Cadm1^{flox/flox}$ mutant mice that were previously crossed to C57BL/6 (Jackson Labs) for four generations. Cadm1Vglut2-Cre mice were crossed to *ob/ob* mice (Jackson Labs). Results were consistent in both genders however data from female mice is not shown.

3.2.3.2 Generation of tetO-Ago2 transgenic mice and induced expression of Ago2

To generate tetO-Ago2 mice, the cDNA containing the coding sequence of a N-terminal FLAG/HA-Tag and of murine Ago2 (a kind gift of G. Meister, University of Regensburg) was cloned into a pTRE-2 vector (Clontech), and the resulting construct was used for microinjections. The offspring was screened for integration of the transgene by PCR, using genomic DNA from tail biopsies. Positive founders were further crossed with Ins-rtTA mice (Nir et al., 2007). Overexpression of Ago2 specifically in the beta cell was achieved by supplementing the drinking water with 5% (w/v) sucrose and 1 mg/ml doxycycline. Double-transgenic animals were screened for degree of overexpression in isolated pancreatic islet after 1 week of treatment with doxycycline.

3.2.3.3 Genotyping of mouse tail biopsies

Mice were weaned at an age of 3 to 4 weeks and separated based on gender with not more than 6 mice per cage. The tail biopsies were digested in 250 µl tail lysis buffer, supplemented with 4 µl Proteinase K (10 mg/ml) and incubated over night at 55 °C. Cell debris was spun down at 12.000 g for 5 min and supernatants were transferred to a new tube. Subsequently, genomic DNA was precipitated by adding 700 µl of Ethanol (100 %) and 15 µl 2.5 M sodium acetate. Precipitated DNA was pelleted for 7 min at 12.000 g, washed once with 500 µl of Ethanol (70 %). The pellet was resuspended in TE buffer.

1x tail lysis buffer:

4 M	Urea
10 mM	EDTA pH 8.0
0.5 %	Sarkosyl
0.1 M	Tris HCl pH 8.0
0.2 M	NaCl

Genotyping-PCR was performed in a 15 µl reaction, using a *Taq*-Polymerase (Life Technologies) and following the manufacturer's instructions. Gene specific Primers are summarized in Table 10.

Table 10: Primer Sequences for Mouse Genotyping

allele	Forward Primer (5'-3')	Reverse Primer (5'-3')
tetO-Ago2	ATCCACGCTGTTTTGACCTC	GAGCAGGAGAAGCAAGAACG
Ago2 fl/fl	TGATCATGGTTGAGGTCTGA	GTGAGCCACTCACTGTGCAC
Cre	GCGGTCTGGCAGTAAAAACTATC	GTGAAACAGCATTGCTGTCACTT
Ins-rtTA	TAGATGTGCTTTACTAAGTCATCGCG	GAGATCGAGCGGGCCCTCGATGGTAG
RIP-Tag	GGACAAACCACAACTAGAATGCAG	CAGAGCAGAATTGTGGAGTGG
miR-375WT	GAGGAAGCTCATCCACCAGAC	GTTCCAGACCTCAGCCCATT
miR-375KO	CCCCGTAATGCAGAAGAAGA	GGTGATGTCCAGCTTGGAGT
Cadm1 WT	CCATGCTATGCTTGCTCATC	AAAGATGATTGCCCATCCAG
Cadm1 KO	AGCATCCCTTTCCACCATAGTTTTCTCTCT	TACCAGGAGGGGAGAAGAGGCCCAGAGC
Vglut2-Cre WT	CGGTACCACCAAATCTTACGG	CATGGTCTGTTTTGAATTCAG
Vglut2-Cre Ki	CGGTACCACCAAATCTTACGG	ATCGACCGGTAATGCAGGCAA
Vgat-Cre WT	CTTCGTCATCGGCGGCATCTG	CAGGGCGATGTGGAATAGAAA
Vgat-Cre Ki	CTTCGTCATCGGCGGCATCTG	CCAAAAGACGGCAATATGGT

3.2.3.4 *In Vivo* Tolerance Tests and *in vivo* insulin release

For glucose and insulin tolerance tests, mice were starved for 6h and interperitoneally (i.p.) injected with glucose in saline at 2 g / kg body weight. For an insulin tolerance test, mice were i.p. injected with 0.75 units insulin per kg body weight. To address insulin sensitivity specifically in the liver, mice were challenged with 2 g / kg body weight pyruvate. For all tolerance tests, plasma glucose levels were measured after 0, 15, 30, 60 and 120 min from tail vein blood.

To measure the release of insulin *in vivo*, mice were injected with glucose at 2 g / kg body weight. Blood was drawn from the tail vein after 0, 2, 5 and 15 min. The blood was spun for 10 min at 10.000 rpm and plasma was transferred to a new tube. Insulin was quantified in 5 µl plasma using an Insulin ELISA (Crystal Chem, Inc.).

3.2.3.5 Hyperinsulinemic-Euglycemic Clamp study

Dr. Andreas Birkenfeld and Sebastian Brachs at the Charité Medical Center in Berlin performed the hyperinsulinemic-euglycemic clamp study (Ayala et al., 2011). Briefly, one week before the clamp study, a catheter was implanted into the right *Vena jugularis*. Animals could recover from the surgical procedure for one week and was characterized by the restoration of the initial body weight. For the clamp experiment mice were starved over night. Hyperinsulinemia was induced by a single dose of 21 mU/kg and a continuous infusion of insulin (3mU/kg/min). Afterwards, the rate of glucose (20 %) infusion was increased until plasma glucose levels reached steady state levels around 100 mg/dl. This point represents the point were infused glucose matches the glucose uptake in insulin responsive tissues and depends greatly on the insulin sensitivity of the animals. Moreover, mice are infused with [3-^{3}H]- Glucose and [1-^{14}C]-2-Deoxyglucose to measure basal glucose metabolism, insulin-stimulated glucose uptake as well as insulin-mediated suppression of gluconeogenesis in the liver.

3.2.3.6 Metabolic Phenotyping of mice using Metabolic Cages

The Phenomaster (TSE Systems) was used to measure the energy expenditure, food intake and locomotor activity. Mice have been acclimatized to the individual housing in an airtight cage for 24 h. During the whole time of recording, animals had ad libitum access to food and water. Locomotor Activity was tracked continuously by light beam breaks in a series of three-dimensional light beams. The respiratory rate (RER) and

energy expenditure (EE) was calculated based on the measured consumptions of oxygen and released carbon dioxide as follows (Speakman, 2013):

$$EE = 3.941 \cdot V(O_2) + 1.106\, V \cdot (CO_2)$$

$$RER = \frac{V\,(CO_2)}{V\,(O_2)}$$

The data was recorded over a period of 96 h in a 1 min-interval. Energy expenditure was normalized by lean body mass that was measured by Dual-energy X-ray absorptiometry (DXA) using a Minispec Model LF90 II (6.5 mHz) (Bruker Scientific Instruments). The animals were fasted from 9 am and drinking water was taken away at 12 am. The body composition was measured between 2 and 3 pm and was performed by Martin Taube at the MDC Berlin, Germany.

3.2.4 Analytical Procedures

3.2.4.1 Insulin and Glucagon Quantification using a Radioimmunoassay

Insulin was quantified in mouse plasma, tissue extracts or cell culture supernatants using the Rat Insulin Radioimmunoassay (RIA) from Millipore (RI-13K) following the manufactures protocol. Briefly, samples were diluted in PBS and incubated with Anti-Insulin antibody and ^{125}I-labeled insulin for 20 to 24 h at 4 °C. Afterwards the antibody-bound insulin was precipitated for at least 20 min at 4 °C and centrifuged for 20 min at 2.500 g at 4 °C. Subsequently, the supernatant was discarded and the gamma ray was measured with the Perkin Elmer WIZARD$_2$® Automatic Gamma Counter. Different insulin standard concentrations were used to calculate the insulin concentration in the sample. Glucagon was measured in a similar fashion using a RIA kit from Millipore (GL-32K).

3.2.4.2 Pancreatic Insulin Content

The pancreatic insulin content was measured in pancreatic lysates. Briefly, mice were scarified at the pancreas was carefully dissected and the weight was measured using an analytical balance. Afterwards the tissue was homogenized in 5 ml of acid ethanol (1.5 % HCl, in 70 % ethanol) for three times 20 sec on ice. The homogenates were stored over night at -20 °C. Next, lysates were vortexed for 30 sec and spun for 15 min at 2000

rpm and 4 °C. The supernatants were transferred to a new tube and neutralized with an equal volume of neutralization buffer (1 M Tris/HCl, pH7.5). The lysates were diluted in PBS and insulin was measured using an Insulin RIA. The content of insulin in the lysates was normalized by pancreatic weight.

3.2.4.3 Western Blotting

For Western Blotting, cells or tissues were homogenized and lysed in an appropriate volume of RIPA lysis buffer and incubated for 10 min on ice. Afterwards, lysates were spun for 20 min at 12.000 rpm and 4 °C and supernatants were transferred to a new tube. The protein concentration was done using a bicinchoninic acid-assay (BCA) and different bovine serum albumin (BSA) concentrations as a standard. 30 µg of protein were boiled for 5 min at 95 °C along with 1x SDS sample buffer. Protein lysates were resolved by SDS polyacrylamide gel electrophoresis (PAGE) using 1x SDS running buffer and subsequently transferred at 4 °C onto nitrocellulose membranes in 1x transfer buffer. The membranes were blocked for 1h with 2.5 % milk in 1x TBST at room temperature and subsequently incubated with respective primary antibodies over night at 4 °C. The next day, membranes were washed 3 times with 1x TBST and incubated with HRP-conjugated secondary antibodies. Followed by another 3 washes, membranes were developed based on chemiluminescence.

1x SDS sample buffer:

310 mM	Tris/HCl
10 %	sodium dodecyl sulfate
50 %	glycerol
5 mM	EDTA
0.05 %	bromophenol blue
5 %	β-mercaptoethanol

1X SDS running buffer:

25 mM	Tris/HCl
192 mM	glycine,
0.1 %	SDS, pH 8.3

1x Transfer Buffer:

25 mM	Tris/HCl, pH 8.4
192 mM	glycine
20 %	methanol

1x TBST:

20 mM	Tris/HCl
137 mM	NaCl, pH 7.6
0.05 %	Tween20

3.2.4.4 Isolation of mRNAs and miRNAs from ribonucleoprotein complexes (RIP)

RIP was performed according to Keene et al. (Keene et al., 2006). In brief, MIN6 cells (~10 million cells) were harvested and the pellet was washed twice with ice cold 1x PBS. The pellet was lysed using an equal volume of polysome binding buffer. After 5 min incubation on ice, lysates were frozen at -80 °C to enhance lysis. Samples were thawed on ice and spun for 15 min at 15.000 g and 4 °C to remove cell debris. Ago2 antibody (WAKO Chemicals) was incubated with Dynabeads Protein G was on a rotating wheel for 20 min at room temperature and beads were washed 5 times with NT2 buffer. Mouse IgG_1 antibody was used as a negative control. Cleared lysates were incubated with beads for 4 h on a rotating wheel at 4 °C. Afterwards, beads were washed 5 times with ice cold NT2 buffer and either boiled in Laemmli buffer or used to extract RNA using the TriZol reagent (Life Technologies).
Incorporated mRNA or miRNA was quantified using qPCR and normalized by RNA from 10 % input lysate. Precipitation of Ago2 was confirmed by Western Blotting.

Polysome lysis buffer:

100 mM	KCl
5 mM	$MgCl_2$
10 mM	HEPES (pH 7.0)
0.5%	NP40
1 mM	DTT
100 units/ml	RNase Out
400 µM	VRC

Protease inhibitor cocktail

NT2 buffer:

50 mM	Tris-HCl (pH 7.4)
150 mM	NaCl
1 mM	$MgCl_2$
0.05%	NP40

3.2.4.5 Confocal Imaging and quantification of beta cell mass

Islet morphometric analysis after intraperitoneal injections of BrdU on four consecutive days (50 µg/g Body Weight, Sigma B5002) was performed on 8 µm sections of paraffin-embedded pancreas approximately 150–200 µm apart. Sections were dewaxed for 20 min at 60 °C, two washes with Xylol and a decreasing row of Ethanol for 5 min each. Afterwards sections were hydrated in 1x PBS and subsequently blocked in 5 % goat-serum in 1x PBST. Afterwards slides were incubates with primary antibodies for insulin, glucagon, BrdU, Ki-67, or TUNEL at 4 °C in a humidified chamber. Next sections were washed 3 times for 5 min with 1x PBS and incubated with the fluorophore-labeled secondary antibodies for 1 h at RT. After another 3 washes for 5 min, cells were embedded in Florescent Mounting Media (DAKO). Cell numbers from all islets in 3–7 sections were counted with ImageJ software from 20x images and normalized by tissue area. Beta cell mass was measured as the ratio of insulin-positive cell area to the total tissue area, multiplied by the weight of the pancreas using Imaris software (Bitplane). For the quantification of BrdU-positive and apoptotic cells, islets from at least 20 islets per slide and 3 different sections per animal were analyzed and normalized by total number of insulin positive cells.

3.2.4.6 Transmission electron microscopy of pancreatic islets

The whole pancreases were dissected, fixated in 4% paraformaldehyde and sliced with a scalpel to smaller sections (1–2 mm) as described previously (Wendt et al., 2012). Samples were incubated in 2.5% glutaraldehyde overnight and treated with 1% osmium tetroxide for 2 h, dehydrated, and then embedded in AGAR 100 (Oxford Instruments Nordiska AB, Sweden). Finally they were cut in 70- to 90-nm sections, put on Cu-grids, and contrasted with uranyl acetate and lead citrate. The samples were examined in a JEM 1230 electron microscope (JEOL-USA. Inc., Peabody, MA), and the micrographs were analyzed with respect to LDCV density and docked LDCV density as previously described (Vikman et al., 2009). The diameter of individual vesicles was determined using Scion Image (NIH freeware). The granule volume density (Nv) and surface density (Ns) were calculated using in-house software programmed in MatLab (version 7x).

3.2.4.7 Tumor burden

The tumor burden of RIP-Tag and 375KO/RIP mice was measured in 12-week-old mice as previously described (Bergers et al., 2003). Briefly, mice were sacrificed and the pancreas was carefully dissected. The dimensions of all tumors were precisely measured using a caliper. The tumor burden is defined as the sum of all tumor volumes within a pancreas. The volume of a tumor was calculated as follows:

$$\text{Volume } [mm^3] = 0.52 \times (\text{width})^2 \times (\text{length})$$

3.2.4.8 Isolation of hippocampal synaptosomes

Synaptosomes were isolated from hippocampi of 12 week-old $Cadm1^{Vglut2\text{-}Cre}$ mice. Mice were sacrificed and hippocampi were immediately homogenized in 2 ml of ice-cold homogenization-buffer with 8 strokes using a Potter Teflon-glass homogenizer on ice. Subsequently, lysates were spun for 10 min at 900 g at 4 °C to remove cell debris and nuclei. The supernatant was transferred to a new tube and spun again at 10.000 g and 4 °C for 15 min. Afterwards, the supernatant was discarded and the resulting P2 fraction, containing crude synaptosomes, was lysed and used for Western Blotting.

Homogenization buffer:

20 mM	HEPES pH 7,4
320 mM	sucrose

3.2.4.9 Electron microscopy analysis

4 pairs of adult, 2 months old WT and $Cadm1^{Vglut2\text{-}Cre}$ littermates were anesthetized and transcardially perfused with 4% formaldehyde and 2.5% glutaraldehyde in PBS. Brains were isolated and postfixed in the same solution overnight at 4°C. After rinsing in PBS, brains were embedded into 5% agar and sliced coronally (200 µm sections) on the vibratome (Leica). Vibratome slices containing hippocampi were postfixed in 1% OsO_4 and 1.5% potassium hexacyanoferrat, followed by dehydration in a methanol gradient and propylene oxide, and flat embedded in epoxy resin. After polymerisation the CA1 pyramidal cell layer and adjacent stratum radiatum were trimmed and ultrathin sectioned. Sections were collected on coated slotted grids and analysed using a Zeiss 900 transmission electron microscope. Synaptic density was determined by the disector

method (Jeffrey et al., 2000). In short, neuropil images with an area of ≈190 µm² from adjacent sections with defined thickness were aligned, a disector grid was superimposed onto the images and the appearance of the postsynaptic density was used as a "counting cap". At least 20 disectors were analysed per animal. Alternatively density of synaptic profiles was estimated in 2D by counting number of profiles in 160 neuropil fields. Dmytro Puchkov performed the embedding, sectioning and image acquisition.

3.2.5 Statistical Methods

All results are expressed as mean ± Standard Error of Mean (SEM). Comparisons between data sets with two groups were evaluated using an unpaired Student's t-test. ANOVA analysis was performed for comparisons of three or more groups. The survival comparison between two groups was done with a log-rank test using the GraphPad Prism software (Version 5). A p-value of less than or equal to 0.05 was considered statistically significant (*, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$).

4 Results

4.1 Characterization of the functional role of Ago2 in the beta cell

The miRNA pathway has been implicated as a mediator of cellular stress responses in order to maintain steady-state physiology, but also plays a role during development and organogenesis (Bushati and Cohen, 2007; Emde and Hornstein, 2014). Ago2 is the most abundant member of the family of Argonaute proteins and a key component of the miRNA machinery (Valdmanis et al., 2012). It mediates the interaction between the miRNA and its targets and facilitates the suppression of gene expression. However, its functional role in the pancreatic beta cell is not characterized.

4.1.1 Generation of conditional Ago2 knockout mouse in the beta cell

In order to address the role of the miRNA pathway in the pancreatic beta cell, a conditional knockout of Ago2 specifically in the beta cell was generated, by crossing *Argonaute*$^{flox/flox}$ mice (O'Carroll et al., 2007) with Ins-Cre mice expressing the Cre Recombinase under the control of the insulin promoter (Herrera, 2000). Conditional Ago2 knockout mice (βAgo2KO) were born at expected Mendelian ratio. The loss of Ago2 was confirmed at protein level by western blotting from isolated islets (Figure 5A). Furthermore, loss of Ago2 resulted in a mild compensatory up-regulation of Ago1. βAgo2KO mice did not show any significant difference in body weight as well as fasted and fed plasma insulin and blood glucose levels (Figure 5B-C).

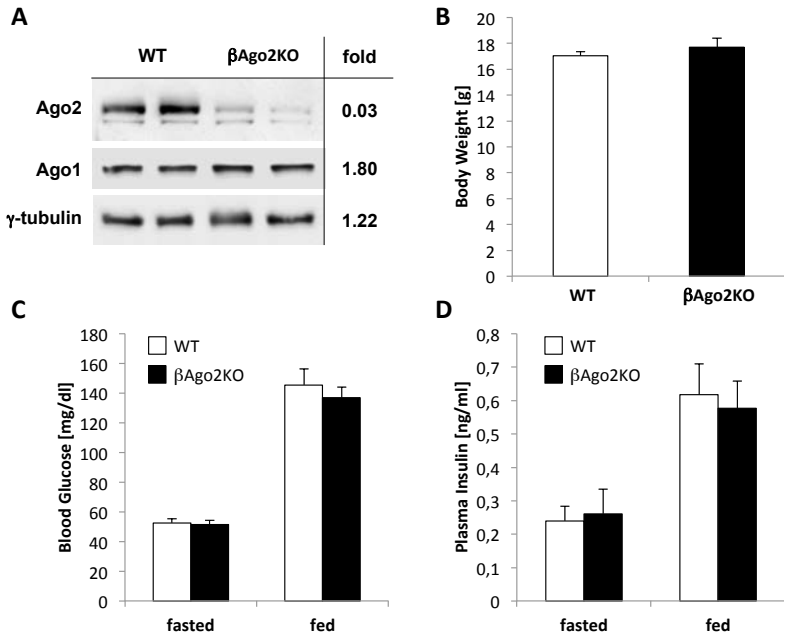

Figure 5: Generation of conditional Ago2 knockout mice in the beta cell.
(A) Western blot analysis of Ago2 and Ago1 from islets of 10-week-old βAgo2KO mice and WT. **(B)** Body Weight from 10-week-old βAgo2KO (n=5-7). **(C)** Fasted and fed plasma insulin levels from 10-week-old βAgo2KO (n=5). **(D)** Fasted and fed blood glucose levels from 10-week-old βAgo2KO (n=5).

4.1.2 Loss of Ago2 results in improved glucose tolerance

In order to further address the functional consequence of genetically ablating Ago2 from beta cells *in vivo*, mice were challenged with glucose. βAgo2KO mice exhibited a significantly improved glucose tolerance test, resulting in faster clearance of glucose (Figure 6A). The Cre-recombinase under the control of the insulin promoter is also mildly expressed in the hypothalamic neurons that are implicated in the regulation of insulin sensitivity in peripheral tissues (Wicksteed et al., 2010). However, an insulin tolerance test in βAgo2KO mice did not show any significant difference, ruling out an unspecific effect of Ago2 deletion in hypothalamic neurons, which may alter glucose tolerance and insulin sensitivity (Figure 6B). Taken together, loss of Ago2 in beta cells results in improved tolerance without affecting insulin sensitivity.

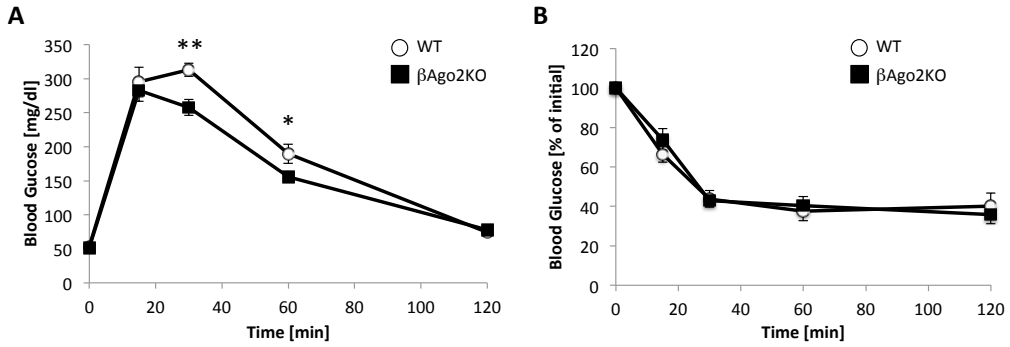

Figure 6: Loss of Ago2 results in improved glucose tolerance without affecting insulin sensitivity.
(A) Blood glucose levels during a glucose tolerance test on 10-week-old βAgo2KO mice and WT littermates (n = 4–5) **(B)** Blood glucose levels during a insulin tolerance test on 14-week-old βAgo2KO mice and WT littermates (n = 7–10).

4.1.3 Ago2 regulates beta cell proliferation

Loss of Ago2 resulted in improved glucose tolerance presumably due to increased insulin output from the pancreatic beta cell and can be explained either due to increased beta cell mass or increased insulin secretion. Therefore the islet morphology and beta cell mass was analyzed in βAgo2KO mice. As shown in Figure 7A, loss of Ago2 did not result in any abnormalities in islet morphology. However, pancreatic beta cell mass was significantly reduced in βAgo2KO mice compared to WT littermates, which was also reflected by decreased number of insulin positive cells per mm^2 of pancreas and reduced pancreatic insulin content (Figure 7B, D and F). In contrast the number of glucagon positive cells, pancreatic glucagon content as well as total pancreatic mass was not changed (Figure 7C, E and G). To address the cause of reduced beta cell mass of βAgo2KO animals, beta cell proliferation and the rate of apoptosis was analyzed. As shown in Figure 7H the number of BrdU positive cells significantly decreased in βAgo2KO animals, while the number of TUNEL positive apoptotic cells was not affected (Figure 7I) Taken together, loss of Ago2 resulted in decreased beta cell mass due to decreased proliferative capacity of the cell without affecting beta cell turnover and the architecture of pancreatic islets.

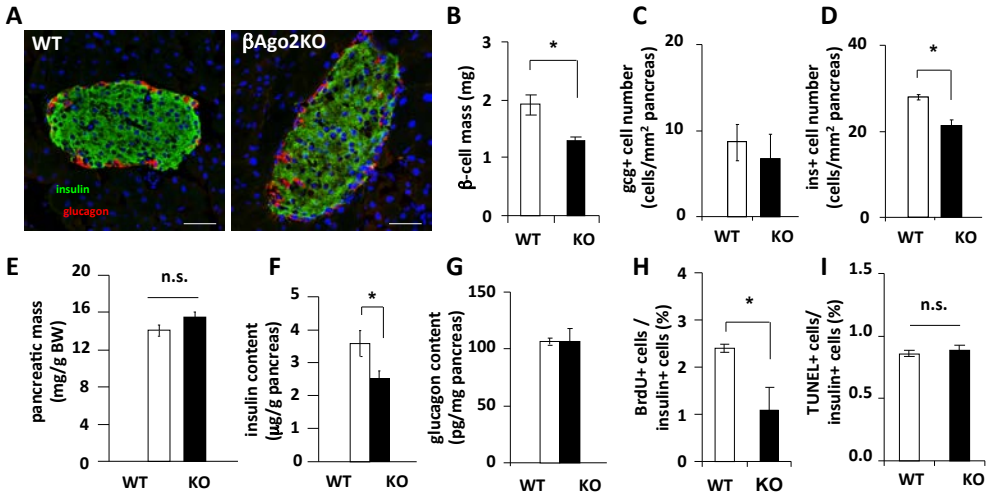

Figure 7: Ago2 regulates pancreatic beta cell mass.
(A) Immunostaining of pancreatic sections from 10-week old βAgo2KO mice and littermates with antibodies to insulin (green) and glucagon (red). Scale bars, 50 µm. **(B)** Beta cell mass analysis of 10-week-old βAgo2KO and WT mice (n = 3). **(C and D)** Morphometric analysis of insulin+ and glucagon+ cells in βAgo2KO and littermates at 10 weeks of age (n = 5–6). **(E)** Pancreatic mass of βAgo2KO and littermates at 10 weeks of age (n = 5). **(F and G)** Pancreatic insulin and glucagon content of βAgo2KO and littermates at 10 weeks of age (n = 5). **(H)** Ratio of BrdU+ and insulin+ cells in βAgo2KO and littermates at 10 weeks of age (n = 5–6). **(I)** Ratio of TUNEL+ and insulin+ cells in βAgo2KO and littermates (n = 3).

4.1.4 Loss of Ago2 results in increased insulin secretion

Since decreased pancreatic beta cell mass in βAgo2KO mice cannot account for the improved glucose tolerance, insulin release was measured *in vivo*. As shown in Figure 8A, loss of Ago2 results in a significantly increased release of insulin after challenge with glucose compared to WT littermate controls. This was further confirmed in vitro using the murine pancreatic beta cell line MIN6. A siRNA mediated knockdown of Ago2 lead to an enhanced release of insulin in response to 25 mM glucose, as shown in Figure 8B. Furthermore, Electron Microscopy showed that the beta cell area of βAgo2KO mice was significantly increased, as well as the total number of insulin secretory granules (ISGs) as volume density (Figure 8C and D). The surface density of docked granules to the plasma membrane was not changed (Figure 8E). In summary, loss of Ago2 resulted in increase of insulin release, partially by an enhanced volume density of the granules.

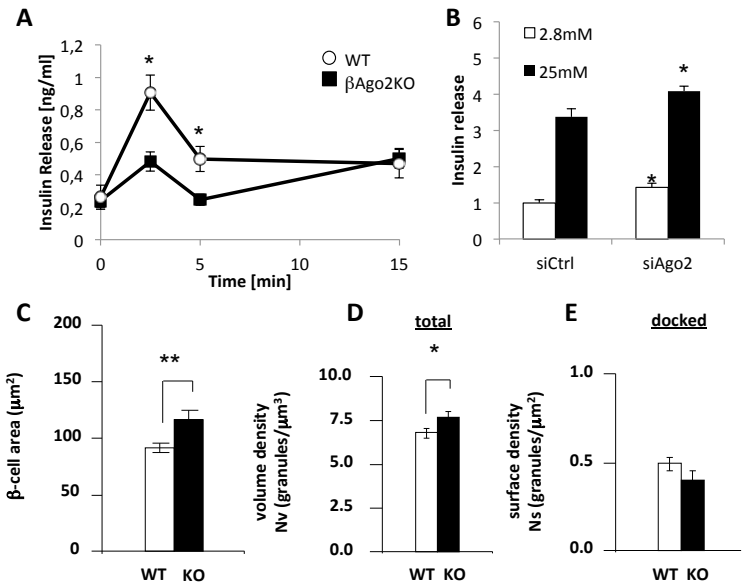

Figure 8: Ago2 regulates the release of insulin.
(A) Plasma insulin after glucose challenge on 12-week-old βAgo2KO mice and WT littermates (n = 4–5). **(B)** Measurement of glucose-stimulated insulin release in MIN6 cells after knockdown of *Ago2* compared to scrambled controls (2.8 mM glucose, white bar; 25 mM glucose, black bar) (n=4). **(C)** Quantification of cell area (µm2) on random beta cells in βAgo2KO and WT from 10 weeks of age (n=5-8). **(D and E)** Quantification of total amount of LDCVs measured as volume density Nv (granules/µm3) and docked large dense core vesicles (LDCVs) measured as surface density Ns (granules/µm2) in βAgo2KO and WT from 10 weeks of age (n=3).

4.1.5 Generation of an inducible transgenic mouse model to overexpress Ago2 in beta cells

In order to gain more insight into the function of Ago2 in the pancreatic beta cell, a novel mouse model was generated, which allowed the inducible overexpression of Ago2 specifically in the pancreatic beta cell. In principle, this model consists of two different transgenes. The first one was acquired from Jackson Laboratories and carries an expression cassette, which drives the expression of the reverse tetracycline controlled trans-activator under the control of the insulin promoter specifically in pancreatic beta cells (Ins-rtTA). The second transgene was generated as follows. The coding sequence of a FLAG/HA-tagged murine Ago2 (kindly provided by Gunter Meister, University of Regensburg) was subcloned into the pTRE2 vector, which contains a tetracycline-responsive element (tetO-FLAG/HA-Ago2). The linearized expression cassette was introduced by pronuclear microinjection in murine blastocysts and implanted in pseudo-pregnant females. Four positive founders were identified by PCR-based genotyping and further bred with Ins-rtTA mice. Double transgenic animals, consisting of the Ins-rtTA

as well as the tetO-FLAG/HA-Ago2 cassette (tetO-Ago2) were used for the experiments and received doxycycline with the drinking water. Mechanistically, rtTA binds only in the presence of doxycycline to the tetO-binding element and drives the expression of FLAG/HA-Ago2 specifically in the pancreatic beta cell (Figure 9A). As shown in Figure 9B, western blot analysis of isolated islets from four different founder animals treated for one week with doxycycline resulted in various degree of over-expression ranging from mild (~1.5 fold; founder #14) to strong (~24 fold; founder #30) over-expression. Founder #14 and #30 were used for further analysis. As shown in Figure 10A-D, random blood glucose levels as well as body weights did not significantly differ after treating these mice for about 3 weeks with doxycycline.

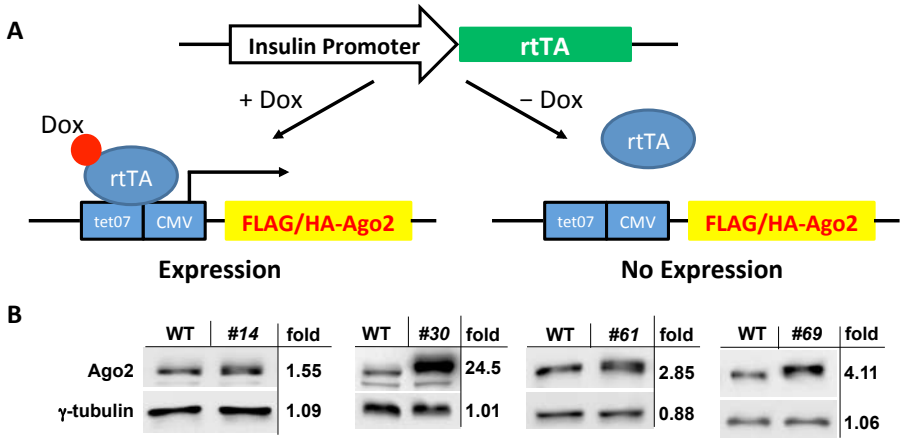

Figure 9: Generation of inducible Ago2 transgenic mouse model.
(A) Schematic overview inducible transgenic mouse model. Briefly, insulin promoter is driving expression of a reverse tetracycline controlled trans-activator, which is driving the expression of FLAG/HA-tagged Ago2 specifically in the beta cell in the presence of doxycycline. **(B)** Western blot analysis of Ago2 in isolated pancreatic islets from four different transgenic founder lines after 1 week of doxycycline treatment.

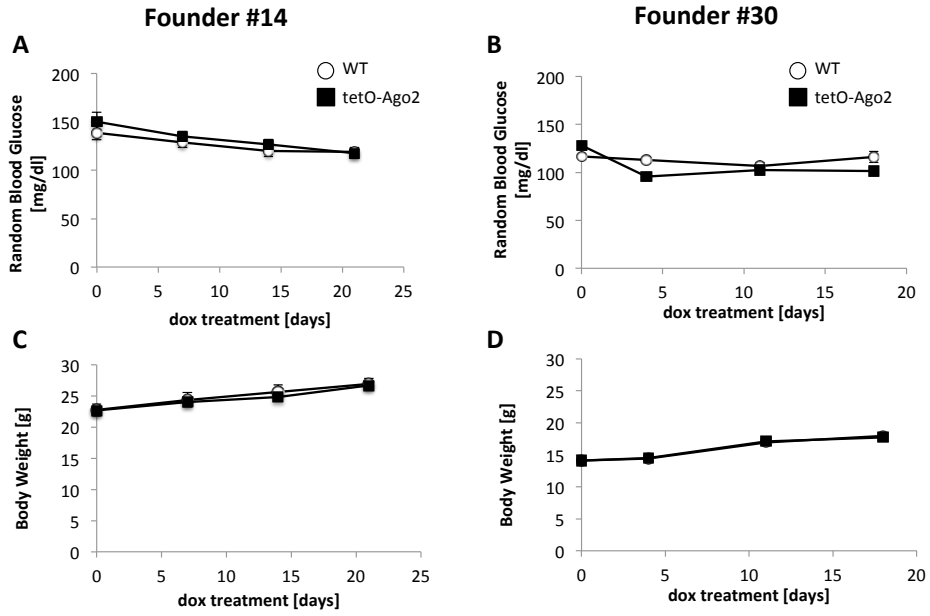

Figure 10: Random glucose levels and body weights of tetO-Ago2 mice
(A and B) Random blood glucose levels of two different tetO-ago2 founder lines over a period of about 3 weeks of doxycycline treatment. **(C and D)** Body weights of two different tetO-ago2 founder lines over a period of about 3 weeks of doxycycline treatment.

4.1.6 Overexpression of Ago2 in the beta cell results in glucose intolerance due to suppression of insulin release

Subsequently, beta cell function was addressed using two different tetO-Ago2 lines. With increasing levels of Ago2 overexpression, tetO-Ago2 mice show impaired glucose tolerance test. Mild overexpression (line #14) showed a trend towards glucose intolerance, however this was not statistically significantly. In contrast, upon high levels of overexpression, tetO-Ago2 mice become glucose intolerant (Figure 11A and B). In line with these observations, the release of insulin worsens with increasing levels of Ago2 expression, as shown in Figure 11C and D. Furthermore, this observation could be confirmed in MIN6 cells, where overexpression of Ago2 resulted in decreased release of insulin (Figure 11E). In order to rule out any effect of perturbed insulin sensitivity in tetO-Ago2 mice, insulin tolerance test was done on line #30. As shown in Figure 11F, overexpression of Ago2 in the pancreatic beta cell did not result in any change of insulin sensitivity. Taken together, transgenic overexpression of Ago2 in beta cells results in decreased insulin release and impaired glucose tolerance.

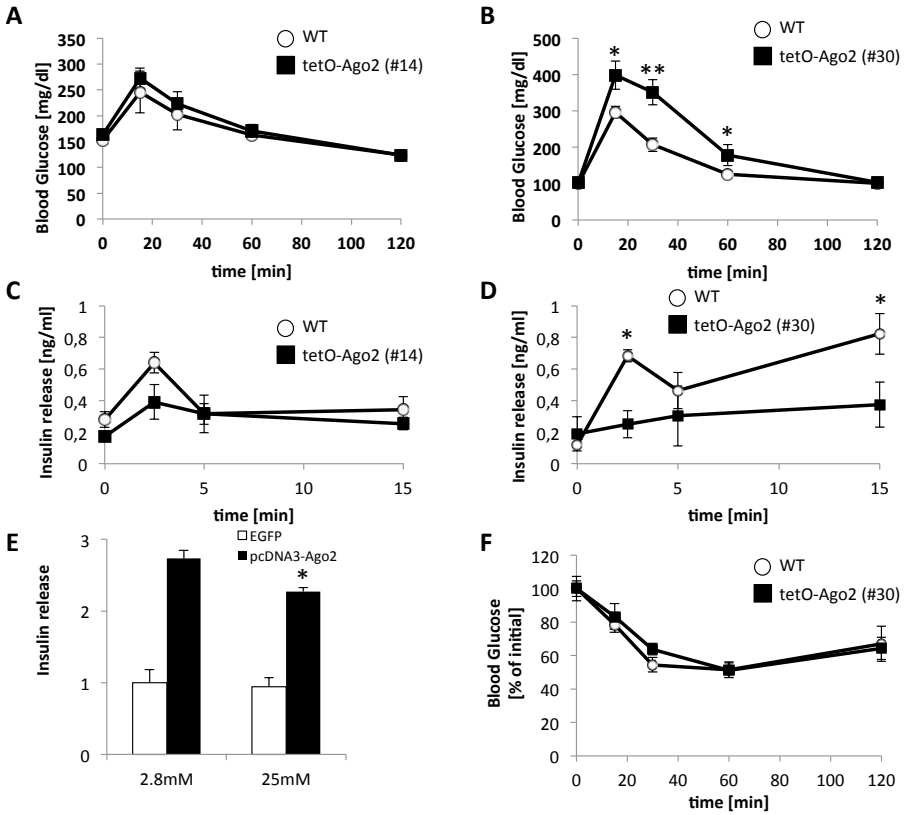

Figure 11: Ago2 regulates the release of insulin.
(A) Blood glucose during a glucose tolerance test (GTT) on 10-week old tetO-Ago2 mice (#14) and WT (n=4-5). **(B)** Blood glucose during a glucose tolerance test (GTT) on 10-week old tetO-Ago2 mice (#30) and WT (n=4-5). **(C)** Plasma insulin levels after glucose infusion on 10-week old tetO-Ago2 mice (#14) and littermates (n=5). **(D)** Plasma insulin levels after glucose infusion on 10-week old tetO-Ago2 mice (#30) and littermates (n=5). **(E)** Measurement of glucose-stimulated insulin release in MIN6 cells after overexpression of Ago2 compared to scrambled controls comparing low (2.8 mM glucose, white bar) and high glucose (25 mM glucose, black bar) (n=4). **(F)** Blood glucose levels during an insulin tolerance test on 14-week-old tetO-Ago2 mice (#30) and WT littermates (n = 5).

4.1.7 Ago2 regulates beta cell mass

As described above, loss of Ago2 expression in beta cell resulted in decreased pancreatic beta cell mass. To address the effect of transgenic overexpression of Ago2, beta cell mass was analyzed in tetO-Ago2 mice (line #30). As shown in Figure 12A and B, tetO-Ago2 mice exhibit increased beta cell mass after 8 weeks of doxycycline treatment compared to wild type littermate controls. In line with this, transgenic overexpression of Ago2 caused an increase in number of BrdU positive cells (Figure 12C). Furthermore, the number of insulin positive cells per pancreatic area is significantly increased, whereas the number of glucagon positive cells did not differ

(Figure 12D and E). In summary, overexpression of Ago2 in pancreatic beta cells results in increased proliferation and beta cell mass.

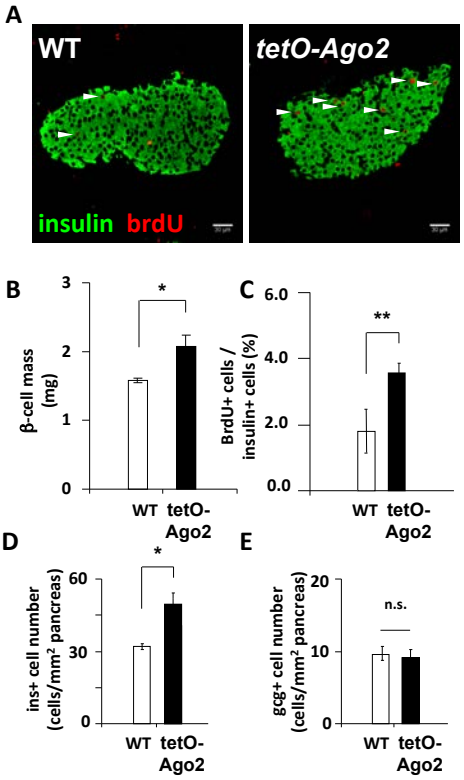

Figure 12: Ago2 regulates pancreatic beta cell mass.
(A) Immunostaining of pancreatic sections from 10-week old tetO-Ago2 mice and littermates with antibodies to insulin (green) and brdU (red). Scale bars, 30 µm. **(B)** Beta cell mass analysis of 10-week-old tetO-Ago2 and WT mice (n = 4). **(C)** Ratio of BrdU+ and insulin+ cells in tetO-Ago2 and littermates at 10 weeks of age (n = 5). **(D)** Morphometric analysis of insulin+ cells in tetO-Ago2 and littermates at 10 weeks of age (n = 5). **(E)** Morphometric analysis of glucagon+ cells in tetO-Ago2 and littermates at 10 weeks of age (n = 5).

4.1.8 Ago2 mediates *miR-375* function

As previously shown (Poy et al., 2004; Poy et al., 2009), loss of *miR-375* results in an increased release of insulin and decreased beta cell mass. Similarly, βAgo2KO mice show a similar phenotype, which is also characterized by a decrease of beta cell mass and improved secretion of insulin. In contrast, overexpression of Ago2 results in the opposite phenotype. This observation suggests that Ago2 is a mediator of *miR-375* function. In order to strengthen this argument, Ago2 was knocked-down in MIN6 cells and predicted targets of *miR-375* were analyzed by western blotting. As shown in Figure 13A, knockdown of Ago2 resulted in a compensatory up-regulation of its family member Ago1. *MiR-375* targets, including *Cadm1*, *HuD*, *Gphn*, and *Rasd1* were significantly up-regulated. This suggests that loss of Ago2 resulted in a decreased suppression of *miR-375* targets. Conversely, overexpression of Ago2 resulted in decreased Ago1 expression and suppression of the aforementioned targets (Figure 13B). To rule out a similar effect of other Argonaute family members, Ago1 was knocked down in MIN6 cells. Here, loss of Ago1 did not result in a significant compensatory regulation of Ago2. Among all the tested targets of *miR-375*, only *Rasd1* was up

regulated. *Cadm1* and *Gphn* expression was not changed, whereas the expression of *HuD* was down regulated (Figure 13C). Next, the interaction of Ago2, *miR-375* and its targets was established by antibody-mediated immunoprecipitation of Ago2 complexes. As shown in Figure 13D, Ago2 was efficiently pulled down from MIN6 cell lysates compared to a control IgG antibody. Furthermore, qRT-PCR could confirm a strong enrichment of *miR-375* (Figure 13D) in immunoprecipitated Ago2. As shown in Figure 13E, pull-down of Ago2 also resulted in a significant enrichment of *Cadm1, Hud* and *Rasd1* mRNAs, as quantified by qRT-PCR. Moreover, overexpression of Ago2 resulted in an increased enrichment of the respective targets, whereas inhibition of *miR-375* resulted in a reduction of Ago2-bound target mRNAs. As a control, the beta cell abundant *Ins1* mRNA was tested, which was shown to be present in Ago2 complexes, however in much lower quantities. Moreover, inhibition of *miR-375* did not result in any significant difference in Ago2-bound *Ins1* mRNA.

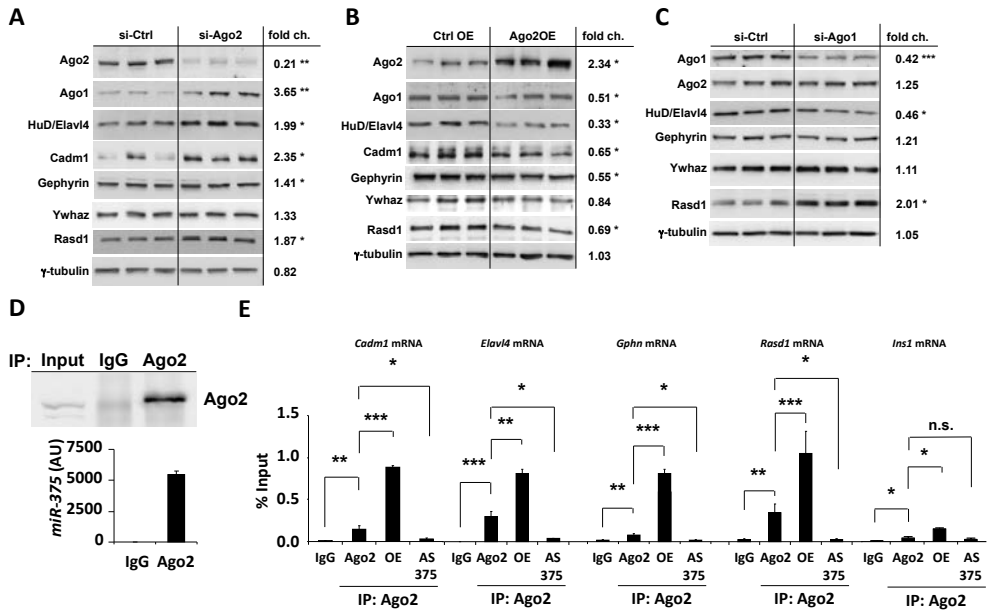

Figure 13: Ago2 mediates miR-375 function.
(A) Western blotting analysis of Ago2, Ago1, Elavl4/HuD, Cadm1, Gephyrin, Ywhaz, and Rasd1 after siRNA-mediated knockdown of *Ago2* compared to scrambled control. **(B)** Western blotting analysis of Ago2, Ago1, Elavl4/HuD, Cadm1, Gephyrin, Ywhaz, and Rasd1 after overexpression of *Ago2* compared to transfection control. **(C)** Western blotting analysis of Ago1, Ago2, Elavl4/HuD, Gephyrin, Ywhaz, and Rasd1 after siRNA-mediated knockdown of *Ago1* compared to scrambled control. **(D)** Detection of Argonaute2 after immunoprecipitation from MIN6 cell lysates with a-Ago2 antibody and quantification of *miR-375* by qRT-PCR after isolation of total RNA from Ago2-associated complexes. **(E)** qRT-PCR analysis of *Cadm1, Elavl4, Gphn, Rasd1 and Ins1* after immunopreciptation of Ago2 from MIN6 cells either untransfected (Ago2), after over-expression of Ago2 (OE), and after inhibition of *miR-375* with antisense oligonucleotides (AS375) (n=4).

Lastly, the functional link between Ago2 and *miR-375* was validated *in vivo*, using isolated pancreatic islets. As shown in Figure 14A islets from βAgo2KO mice show an increased expression of *miR-375* targets, including Cadm1, HuD, Gphn and Rasd1. In contrast overexpression of Ago2 in tetO-Ago2 animals resulted in significant decrease of targets, as shown by western blot and qPCR (Figure 14B and C). Taken together, Ago2 is a mediator of *miR-375* function and altered expression levels of Ago2 can regulate *miR-375* targets. Furthermore Ago1 doesn't exhibit a similar function, which highlights the role of Ago2 as a crucial mediator of the miRNA pathway in the beta cell.

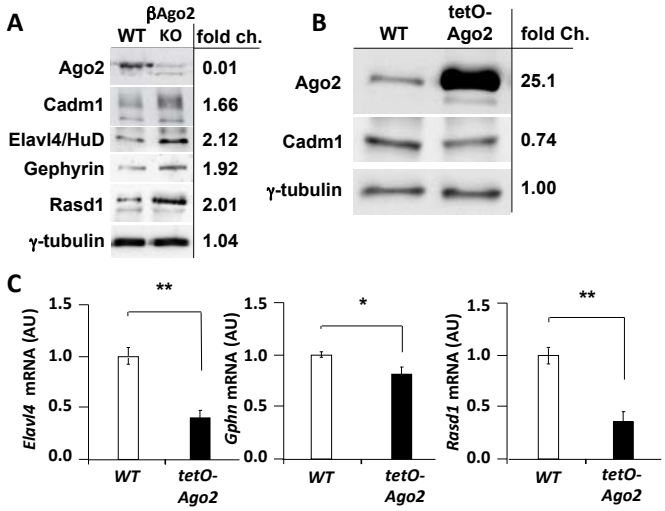

Figure 14: Ago2 mediates *miR-375* function *in vivo*.
(A) Representative western blot analysis of *miR-375* targets in isolated pancreatic islets of βAgo2KO mice and WT littermates **(B)** Representative western blot analysis of Cadm1 in isolated pancreatic islets of tetO-Ago2 mice (line #30) and WT littermates **(C)** qRT-PCR analysis of *Elavl4*, *Gphn* and *Rasd1* in isolated pancreatic islets of tetO-Ago2 and WT littermates (n=4).

4.2 Role of *miR-375* and Ago2 in the regulation of beta cell mass in a mouse model of islet hyperplasia

The miRNA pathway has been shown to be a mediator of cellular stress responses (Emde and Hornstein, 2014). Hence, the role of miRNA pathway becomes usually only apparent, when the cell or an organ readjusts to a new environment. In order to address this phenomenon in more detail, both the miR-375KO and the βAgo2KO mouse models were crossed to the RIP-Tag mouse, a model to study the proliferation of the beta cell. This model is well established and was initially described to study the formation of tumors (Hanahan, 1985). The rat insulin promoter drives the expression of the large T Antigen (Tag), an oncogene, specifically in pancreatic beta cells. This results in a well-defined onset of tumor genesis, which is characterized by the formation of hyperplastic

islets (6 to 8 weeks of age), followed by increased vascularization, the appearance of angiogenic islets (8 to 12 weeks) and the formation of solid tumors (≥14 weeks of age) (Du et al., 2007).

The data from Ago2 loss and gain of function studies in mice as well as in MIN6 cells indicates Ago2 as a mediator of miR-375 function. In order to strengthen this hypothesis, βAgo2KO mice were crossed to RIP-Tag mice (Ago2KO/RIP mice). As shown in Figure 15A, Ago2/RIP mice show a decrease in plasma insulin levels compared to control RIP-Tag mice. Furthermore, survival was significantly prolonged in these animals (Figure 15B).

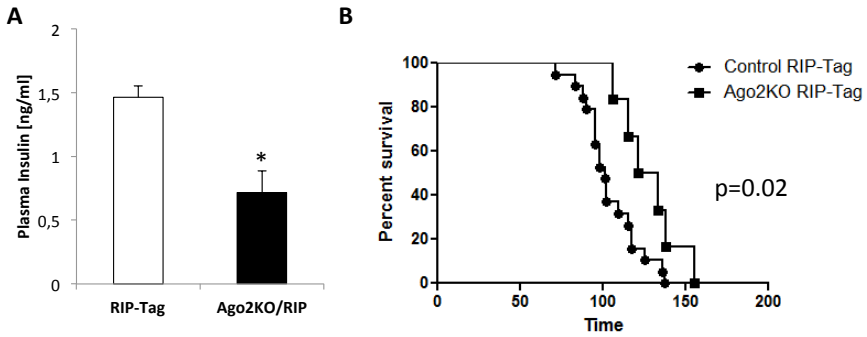

Figure 15: Loss of Ago2 improves survival of RIP-Tag mice.
(A) Plasma insulin levels of RIP-Tag and Ago2KO/RIP animals at 8 weeks of age (n=4-5) **(B)** Survival of RIP-Tag and 375KO/RIP mice (n=6-14).

This observation suggests that loss of Ago2 decreases proliferation in RIP-Tag mice and was further validated by measuring pancreatic beta cell mass in Ago2KO/RIP mice. Loss of Ago2 resulted in a significant decrease of beta cell mass as shown in Figure 16A and B.

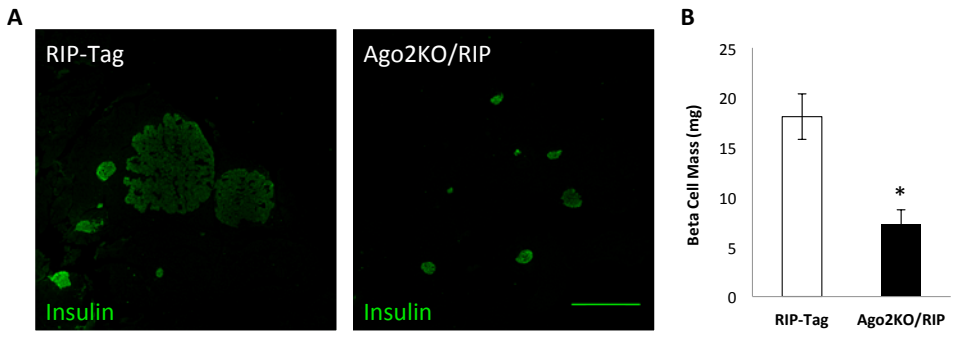

Figure 16: Loss of Ago2 decreases beta cell mass of RIP-Tag mice.
(A) Representative immunostaining of pancreatic sections from 10-week old Ago2KO/RIP mice and RIP-Tag littermates with antibodies to insulin (green). Scale bars, 1000 μm. **(B)** Beta cell mass analysis of 10-week-old Ago2KO/RIP and RIP-Tag littermate controls (n = 3-5).

Previous studies from the groups of Lammert and Powers illustrated an important contribution of intra-islet blood vessels to beta cell growth and function (Lammert et al., 2001; Reinert et al., 2013). Furthermore, Hanahan and colleagues could show that beta cell derived Vegf is critical to regulate angiogenesis in RIP-Tag mice and facilitates growth (Inoue et al., 2002). In order to test if loss of Ago2 in RIP-Tag mice resulted in any alteration in blood vessel density in islets and limits proliferation, vascularization was measured. As shown in Figure 17A and B, loss of Ago2 in RIP-Tag mice did not result in any alteration in blood vessel density. Taken together, loss of Ago2 in RIP-Tag mice resulted in decreased beta cell mass and hyperinsulinemia which resulted in improved survival. The vascularization of islets is not affected and does not contribute to reduced growth.

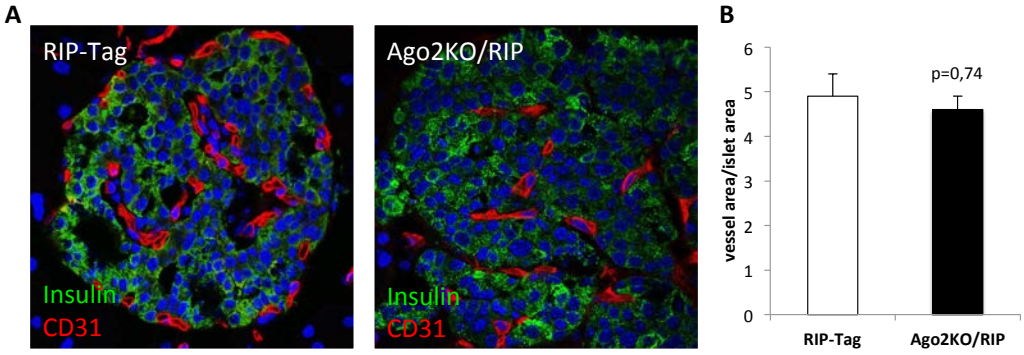

Figure 17: Loss of Ago2 does not alter vascularization in RIP-Tag mice.
(A) Representative immunostaining of pancreatic sections from 10-week old Ago2KO/RIP mice and RIP-Tag littermates with antibodies to insulin (green) and endothelial marker CD31 (red). **(B)** Blood vessel density of 10-week-old Ago2KO/RIP and RIP-Tag littermate controls (n = 3-5).

4.2.1 *miR-375* regulates beta cell mass in RIP-Tag mice

Ago2 incorporates different miRNAs that have different target genes. Hence, perturbing Ago2 expression may also interfere with the function of multiple miRNAs. In order to further validate the functional link between *miR-375* and Ago2, we deleted *miR-375* from RIP-Tag mice (375KO/RIP). In line with previous observations in Ago2KO/RIP mice, 375KO/RIP animals exhibit a delayed reduction in blood glucose levels and shows rescued plasma insulin levels compared to RIP-Tag mice (Figure 18A and B). Moreover, 375KO/RIP mice show a decrease in pancreatic insulin content, suggesting that similar to Ago2KO/RIP mice, loss of *miR-375* results in decreased beta cell mass (Figure 18C). Lastly, in line with previous studies of 375KO mice, deletion of miR-375 in RIP-Tag mice causes an increase in pancreatic glucagon content (Poy et al., 2009) (Figure 18D).

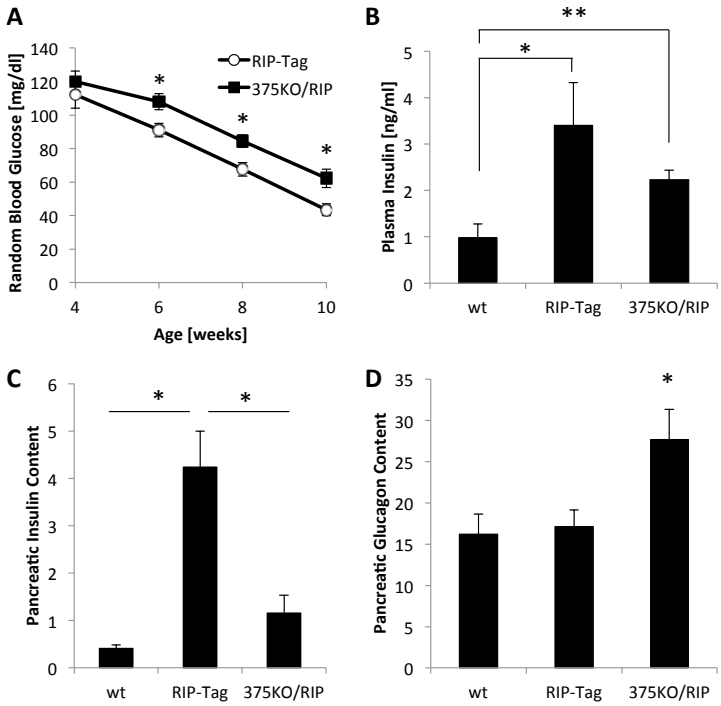

Figure 18: Loss of *miR-375* improves glucose homeostasis in RIP-Tag mice.
(A) Random blood glucose levels of RIP-Tag and 375KO/RIP animals between 4 and 10 weeks of age (n=8) **(B)** Plasma insulin levels of RIP-Tag and 375KO/RIP animals at 8 weeks of age (n=5) **(C)** Pancreatic insulin content of RIP-Tag and 375KO/RIP animals at 12 weeks of age (n=5). **(D)** Pancreatic glucagon content of RIP-Tag and 375KO/RIP animals at 12 weeks of age (n=5).

RIP-Tag mice die due to the formation of tumors and hypoglycemia. Hence, we addressed tumor burden and the survival of 375KO/RIP. As shown in Figure 19A, loss of miR-375 resulted in decreased tumor burden in RIP-Tag mice at an age of 12 weeks. Moreover, we could show that the tumor burden was decreased due to smaller tumor size rather than the tumor number (Figure 19B and C). In line with observations in Ago2KO/RIP mice, decreased beta cell mass in 375KO/RIP mice resulted in significantly improved survival of the animals compared to control RIP-Tag mice (Figure 19D). In summary, loss of miR-375 in RIP-Tag mice reduces proliferation of beta cells and prolongs the survival of these animals.

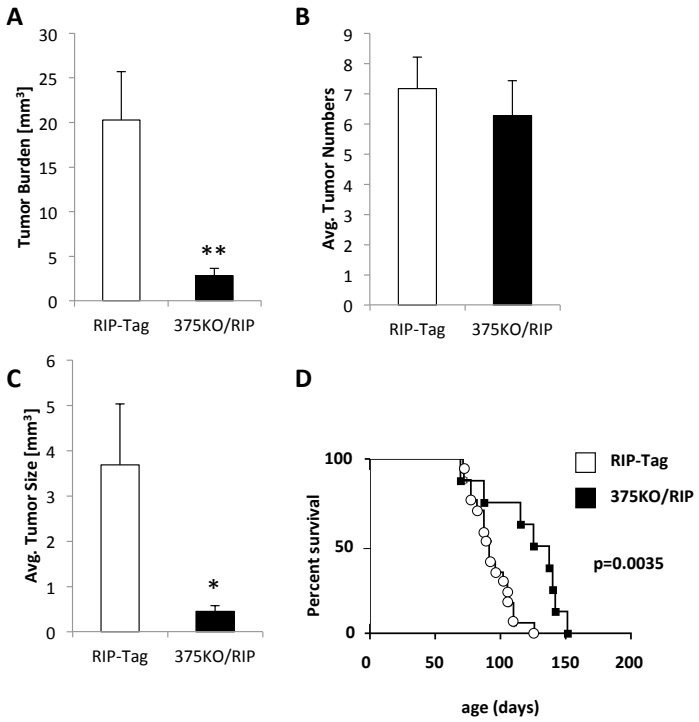

Figure 19: Loss of *miR-375* improves survival of RIP-Tag mice.
(A) Tumor burden of RIP-Tag and 375KO/RIP animals at 12 weeks of age (n=6) **(B)** Average tumor numbers of RIP-Tag and 375KO/RIP animals at 12 weeks of age (n=6) **(C)** Average tumor size of RIP-Tag and 375KO/RIP animals at 12 weeks of age (n=6) **(D)** Survival of RIP-Tag and 375KO/RIP mice (n=8-12).

4.3 Functional characterization of target genes of *miR-375*

4.3.1 Target genes of *miR-375* regulate insulin release

MiR-375 has been shown to be among the most abundant miRNAs in the pancreatic beta cell and regulates the release of insulin as well as beta cell mass (Poy et al., 2004; Poy et al., 2009). These studies could also identify targets of *miR-375*, including *Mtpn, Gphn, Cadm1* and *Elavl4*. However, little is known about the biological role of these targets and how they contribute to mediate the function of *miR-375* by regulating the release of insulin and proliferation of the beta cell.

In order to study the contribution of various target genes to the release of insulin in response of glucose, insulin secretion assays were performed in MIN6 cells. Therefore, 10 previously validated and predicted target genes were down regulated using a pool of 4 different siRNAs. An anti-sense molecule for *miR-375* (Contra375) and knockdown of Glucokinase (*Gck*) served as positive and negative controls, respectively. The knockdown was confirmed by quantitative real-time PCR (Figure 20A-L).

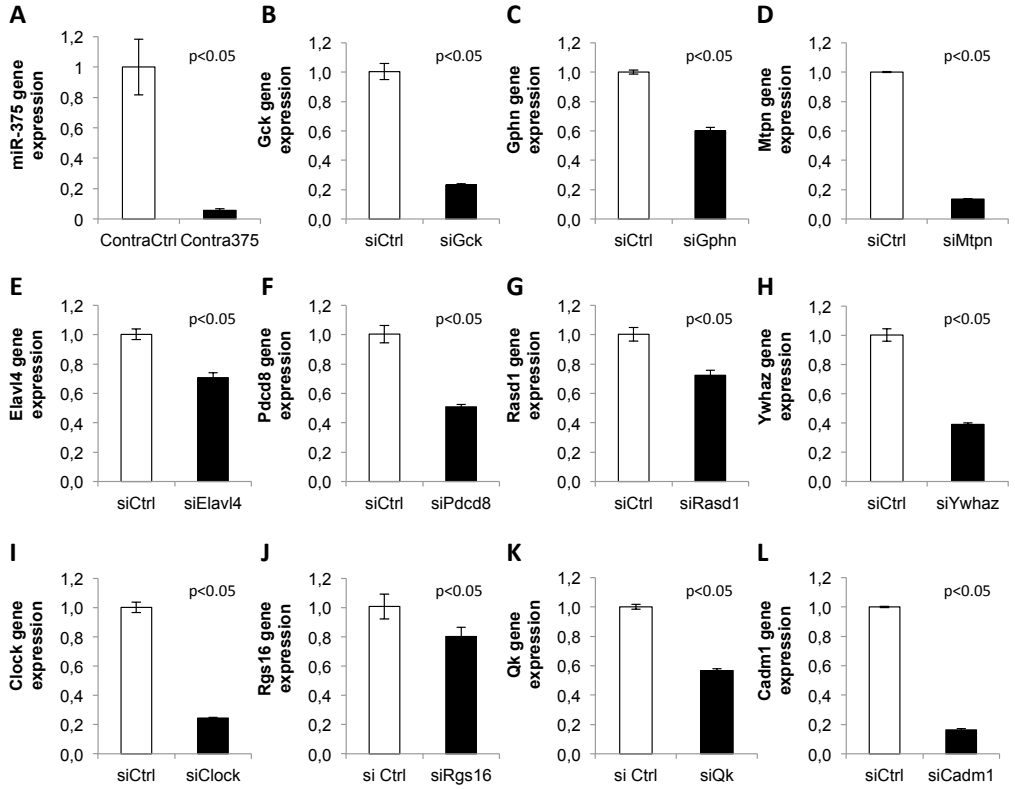

Figure 20: Confirmation of knock-down of *miR-375* target genes.
(A) qRT-PCR analysis after ContraMIR-mediated knockdown of *miR-375* in MIN6 cells compared to control 48 h post-transfection (n=3). **(B-L)** qRT-PCR analysis after siRNA-mediated knockdown of *Gck*, *Gphn, Mtpn, Elavl4, Pdcd8, Rasd1, Ywhaz, Clock, Rgs16, Qk* and *Cadm1* in MIN6 cells compared to scrambled control 48 h post-transfection (n=3).

As previously shown, inhibition of *miR-375* resulted in hyper-secretion of insulin, whereas knockdown of Gck resulted in decrease of insulin release (Figure 21A and B) (Poy et al., 2004; Tattikota et al., 2013). Three of the 10 tested target genes (*Elavl4, Rgs16* and *Qk)* did not show any significant difference in the release of insulin in response to glucose (Figure 21E, J and K). The majority of the target genes, including *Gphn, Mtpn, Pdcd8, Rasd1, Ywhaz* and *Clock* resulted in a decreased secretion of insulin (Figure 21C-D, F-I, L). Only knockdown of *Cadm1* caused hyper-secretion of insulin. Taken together, the majority of the tested *miR-375* target genes regulate the release of insulin. However, these genes can contribute as negative as well as positive regulators of insulin secretion.

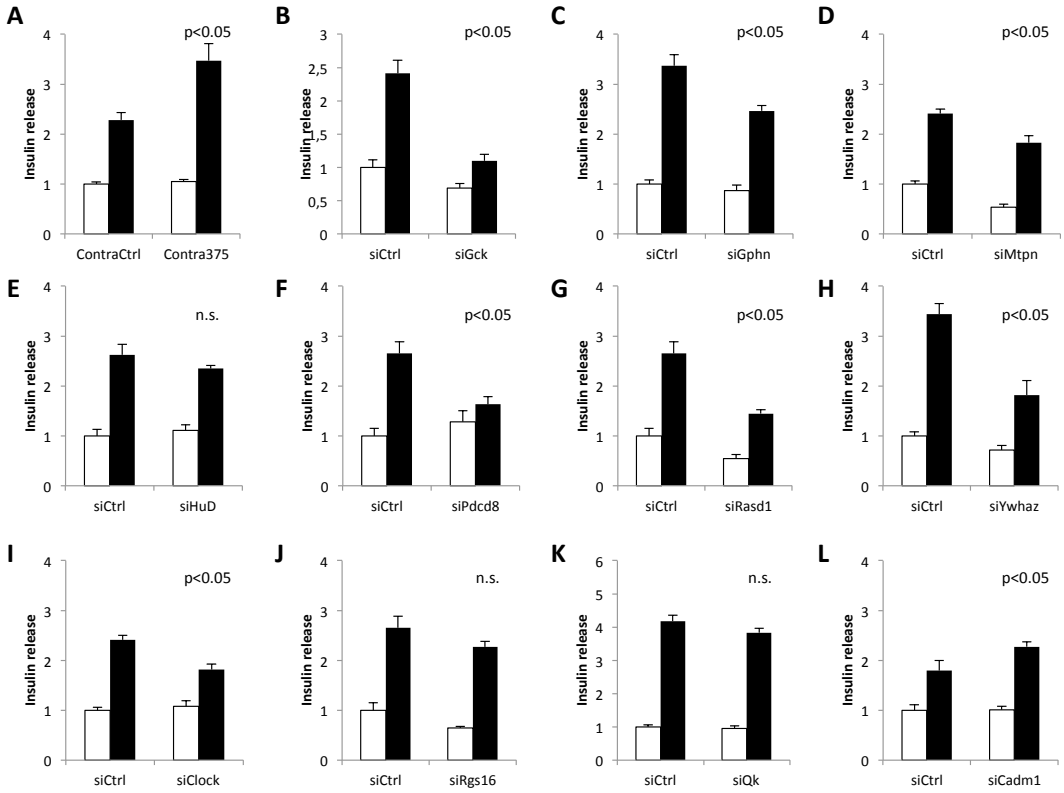

Figure 21: Target genes of *miR-375* regulate insulin release.
(A) Measurement of glucose-stimulated insulin release in MIN6 cells after knockdown of *miR-375, Gck*, *Gphn, Mtpn, Elavl4, Pdcd8, Rasd1, Ywhaz, Clock, Rgs16, Qk* and *Cadm1* compared to scrambled controls comparing low (2.8 mM glucose, white bar) and high glucose (25 mM glucose, black bar) (n=4).

4.3.2 Identification of the pancreatic beta cell and islet secretome

The pancreatic beta cell is recognized as the sole source of insulin. However, little is known about additional secreted proteins, which could serve the inter-organ communication and contribute to glucose homeostasis (Imai et al., 2008; Saltiel and Kahn, 2001). Furthermore, the function of the pancreatic islet also depends on cell-cell interactions within the islet, for example between neighboring beta cells, beta cells and endothelial cells as well as beta to neuronal cell communication (Eberhard and Lammert, 2009). In order to identify secreted proteins from the beta cell, which might be involved in these interactions, a cell culture based model was established using mass spectrometry (Tattikota et al., 2013). This model was used to identify about 50 proteins, which are co-secreted in response to glucose. This model was also used study the effect of inhibition of *miR-375* on the glucose-stimulated release of proteins. As depicted in Figure 22A, inhibition of *miR-375* resulted in hyper-secretion of about 50 proteins at high glucose conditions. This is in good agreement with the previously identified

secretome signature. Moreover, seed enrichment analysis of the secreted factors did not show any significant change for genes containing a *miR-375* seed (Figure 22B). Likewise, we tested the impact of Cadm1 on the MIN6 secretome. In line with the aforementioned secretion experiment, siRNA mediated knockdown of *Cadm1* resulted in hypersecretion of the secretome signature, without significantly altering its composition (Figure 22C). This further underlines the inhibitory function of Cadm1 on the regulation of secretion in beta cells. Furthermore, we isolated pancreatic islets from WT and RIP-Tag mice and identified its secretome. The released proteins in response to 25 mM glucose were normalized by the secretome of heavy labeled MIN6 cells. Interestingly, many proteins, which are released from MIN6 cells, can be also found in isolated islets from WT and RIP-Tag, underling the resemblance of the MIN6 and islet secretome signature. Furthermore, we could detect several proteins that may be involved in the regulation of proliferation of beta cells, including Sez6l, Ambp, Igf2 and Igf2r (Figure 22D). Taken together, inhibition of *miR-375* results in an overall increase of secretion rather than affecting a limited number of proteins. Moreover, Cadm1 has been shown to be a negative regulator of secretion in the pancreatic beta cell. Lastly, inducing proliferation in the beta cell by overexpression of an oncogene, results in altered release of proteins that are involved in the regulation of growth pathways and might contribute to islet hyperplasia.

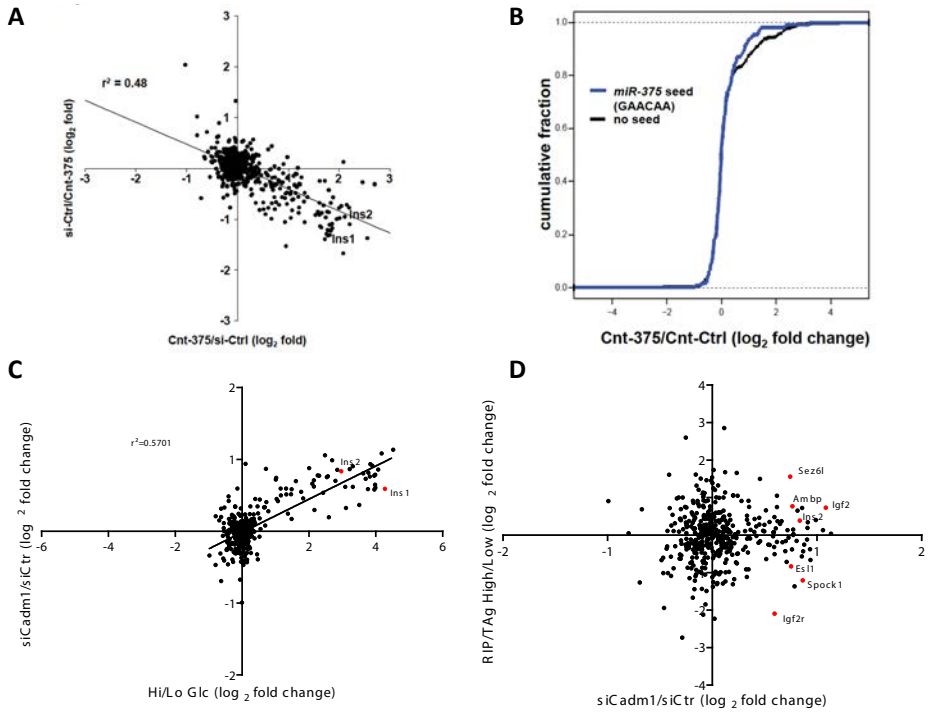

Figure 22: *miR-375* and Cadm1 regulate the pancreatic beta cell secretome.

(A) secretome experiment exhibiting comparison of quantitative analysis of proteins present in supernatant of MIN6 cells after inhibition of *miR-375* and incubation in 25 mM glucose (labeled in heavy SILAC media) and the corresponding label swap experiment. **(B)** Seed enrichment analysis of genes encoding proteins detected in secretome experiments after either inhibition of *miR-375* (Cnt-375). **(C)** Secretome experiment comaring proteins present in supernatant of MIN6 cells after knockdown of *Cadm1* and incubation in 25 mM glucose (labeled in heavy SILAC media) and the corresponding label swap experiment. **(D)** Secretome experiment comparing proteins present in supernatant of MIN6 cells after inhibition of *Cadm1* and incubation in 25 mM glucose (labeled in heavy SILAC media) and supernatants of RIP-Tag islets and incubation in 25 mM Glucose (labeled in light SILAC media).

4.3.3 The *miR-375* target *Cadm1* regulates beta cell mass

In order to understand the functional link between *miR-375* and its targets in more detail, we addressed the role of the cell adhesion molecule 1 (Cadm1) in the regulation of glucose and energy homeostasis in more detail (Poy et al., 2009). *Cadm1* is targeted by *miR-375* and was previously discovered as a negative regulator of growth that is frequently silenced in lung cancer due to promoter methylation (Kuramochi et al., 2001; Murakami, 2002). However, the pathway, which mediates its tumor suppressive function, is not completely understood. Recent studies could show that Cadm1 undergoes membrane shedding and the released intracellular domain migrates to the mitochondria and promotes apoptosis (Inoue et al., 2014; Mimae et al., 2014; Nagara et al., 2012). To further validate the relation between *miR-375* and its target Cadm1, we compared its expression in isolated islets from RIP-Tag and 375KO/RIP islets. As shown in Figure 23A, loss of miR-375 resulted in increased expression of Cadm1.

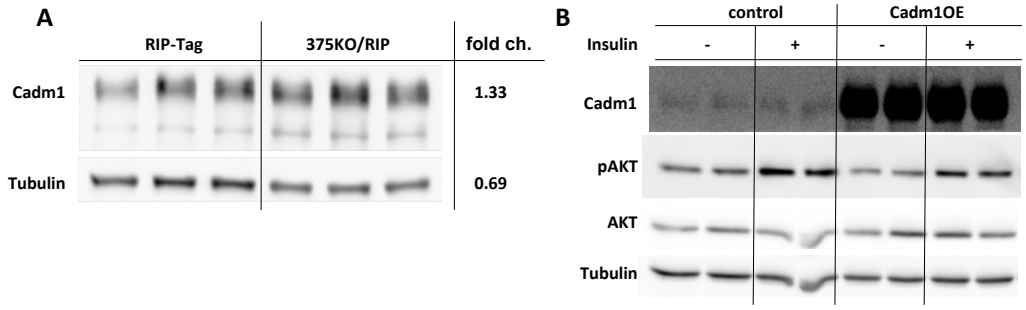

Figure 23: *miR-375* targets *Cadm1* in RIP-Tag mice and regulates the Akt signaling pathway.
(A) Western Blot analysis of 8-week-old RIP-Tag and 375KO/RIP islets. **(B)** Western Blot analysis of Cadm1, phospho-Akt, Akt and Tubulin in MIN6 cells after overexpression of Cadm1 and stimulation with 100 nM Insulin.

Furthermore, previous unpublished observations of our group suggest a potential role of Cadm1 in the mediation of the Insulin Receptor-Akt Signaling pathway, which is

crucial to mediate growth (Taniguchi et al., 2006). To further validate this hypothesis, Cadm1 was overexpressed in MIN6 cells and cells were treated for 30 min with 100 nM Insulin after an overnight starvation. As shown in Figure 23B, overexpression of Cadm1 attenuates the Insulin mediated activation of Akt phosphorylation at serine 473, an indicator for the active Akt signaling pathway.

In order to study the function of Cadm1 in the pancreatic beta cell *in vivo*, Cadm1 total knockout mice (cadm1KO) were obtained and characterized. Previously unpublished data showed that loss of Cadm1 results in improved glucose tolerance test, which is at least partially mediated by increased insulin release from pancreatic islets. To study the impact of Cadm1 on cell proliferation in beta cells morphometric analysis of pancreatic sections was done. As shown in Figure 24A, cadm1KO mice show a significant increase in beta cell mass, an increased number of insulin positive cells per mm^2 pancreas and increased number of BrdU positive cells (Figure 24B and C). In contrast, we did not observe any change in the number of glucagon positive alpha cells (Figure 24D).

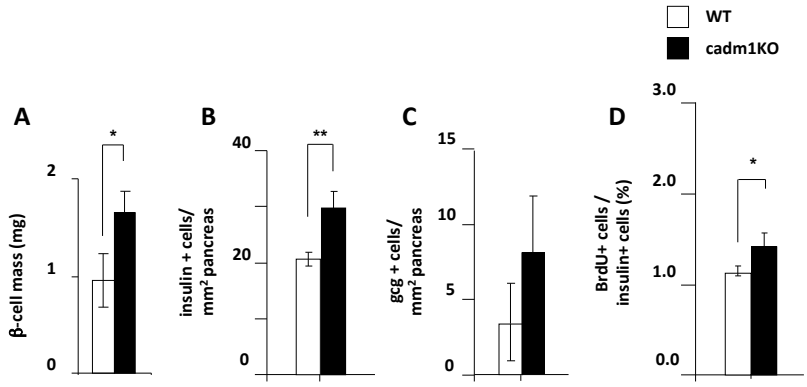

Figure 24: Cadm1 regulates panceratic beta cell mass.
(A) Beta cell mass in 18-week-old cadm1-knockout mice and littermates (n = 4). **(B - C)** morphometric analysis of insulin+ and glucagon+ cells in 18-week-old cadm1-knockout mice and littermates (n = 4). **(D)** Ratio of BrdU to insulin+ cells in 18-week-old cadm1KO mice and littermates (n = 4).

Lastly, cadm1KO mice were crossed to RIP-Tag mice (Cadm1KO/RIP) to test if loss of Cadm1 exacerbates beta cell proliferation. As shown in Figure 25A Cadm1KO/RIP mice showed significant lower blood glucose levels compared to control RIP-Tag mice. Furthermore, survival of Cadm1KO/RIP mice was shortened, though not significantly (Figure 25B). Taken together, *miR-375* targets the cell adhesion molecule Cadm1, which is involved in insulin signaling and regulates beta cell proliferation.

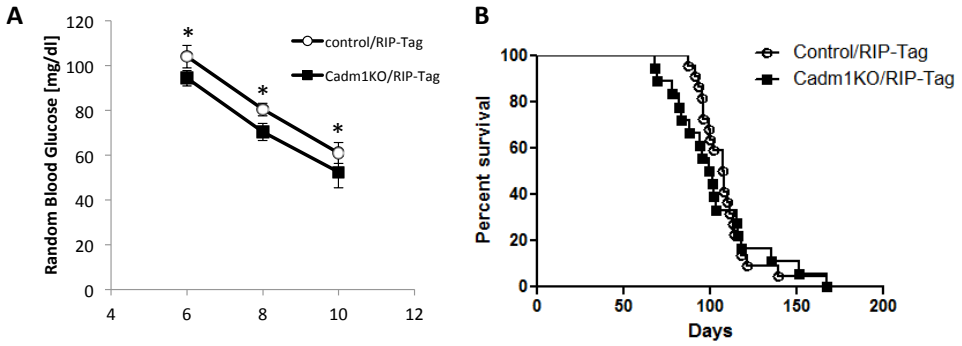

Figure 25: Loss of Cadm1 in RIP-Tag mice exacerbates tumor formation.
(A) Random Blood glucose levels of Cadm1KO/RIP and RIP-Tag mice at different ages (n=6-8) **(B)** Survival of RIP-Tag and Cadm1KO/RIP mice (n=17-18).

4.4 Cadm1 mediates glucose homeostasis in response to changes in nutrient states

4.4.1 Cadm1 expression is regulated in response to variations in nutritional states

The pancreatic beta cell is capable to rapidly adapting to changes in insulin demand and responses to changes in nutritional state (Leibowitz et al., 2008; Sachdeva and Stoffers, 2009). In order to test whether the growth regulator Cadm1 responds to changes in nutrients, expression levels were analyzed in response to a serum starvation. As shown in Figure 26A, Cadm1 expression levels decrease in response to a 16h serum starvation in MIN6 cells, both in control as well as cells overexpressing Cadm1. In addition to its expression in the pancreatic islet, Cadm1 is also strongly expressed throughout the brain where it mediates synaptic interactions (Biederer et al., 2002). To test whether Cadm1 is also regulated in the brain in response to nutritional states, its expression was analyzed in the hippocampus of two different mouse models. The ob/ob mouse is a well-established model in the field of metabolic research and carries a point mutation in the *leptin* gene that disrupts its expression (Zhang et al., 1994). Leptin is a hormone, which is secreted by the adipose tissue and is known to suppress food intake. Loss of leptin expression in ob/ob mice results in abnormal food intake and the development of symptoms of the metabolic syndrome, including obesity, insulin resistance, hyperglycemia and hyperlipidemia (Fellmann et al., 2013). As shown in Figure 26B, Cadm1 levels are up regulated in the hippocampal protein lysates of ob/ob mice. In contrast, after feeding ob/ob mice a ketogenic diet, which was shown to improve sensitivity (Badman et al., 2009), Cadm1 expression levels normalized comparable to

the levels of WT mice, receiving a standard chow diet. Taken together, Cadm1 expression levels respond to changes in nutritional states.

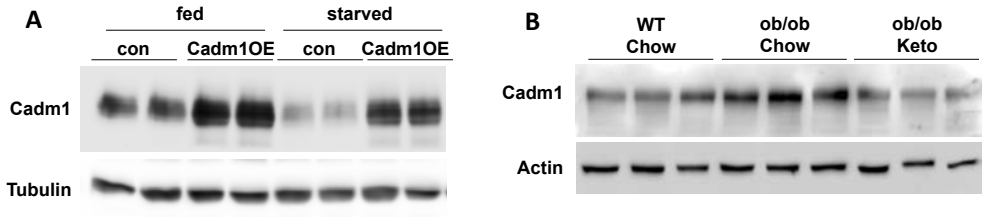

Figure 26: Cadm1 is regulated in response to nutritional changes.
(A) Western Blot analysis of Cadm1 after serum starvation in MIN6 cells overexpressing Cadm1. **(B)** Western Blot analysis of Cadm1 in hippocampus of WT, ob/ob and ob/ob mice receiving a ketogenic diet.

4.4.2 Conditional deletion of Cadm1 in excitatory and inhibitory neurons

In light of the regulation of Cadm1 in the brain due to changes in nutritional state, Cadm1 was deleted in two opposing neuronal populations to further address its contribution to the regulation of energy homeostasis. While excitatory neurons (Vglut2-positive) release glutamate as a neurotransmitter and are abundant in the cortex as well as the hippocampus, inhibitory neurons (Vgat-positive), which release GABA as neurotransmitter, are predominantly present in the central amygdala and some nuclei within the hypothalamus (Vong et al., 2011). Figure 27A and B represent immunofluorescence images of Vglut2-Cre and Vgat-Cre mice crossed to ZsGreen reporter mice (Madisen et al., 2010), in which the reporter is only expressed in Cre-expressing cells. Vglut2-Cre expression is most abundant in the cortex and hippocampus. In contrast Vgat-Cre expression was found mainly in the striatum and some nuclei of the hypothalamus. Furthermore, conditional knockout mice were generated ($Cadm1^{Vglut2}$ and $Cadm1^{Vgat}$). Figure 27C shows the confirmation of Cadm1 ablation in the cortex and hippocampus of $Cadm1^{Vglut2}$ mice, while the olfactory bulb did not show any significant change in Cadm1 expression. In contrast, the strongest decrease in Cadm1 levels was observed in striatum and olfactory bulb of $Cadm1^{Vgat}$ mice, as shown in Figure 27D.

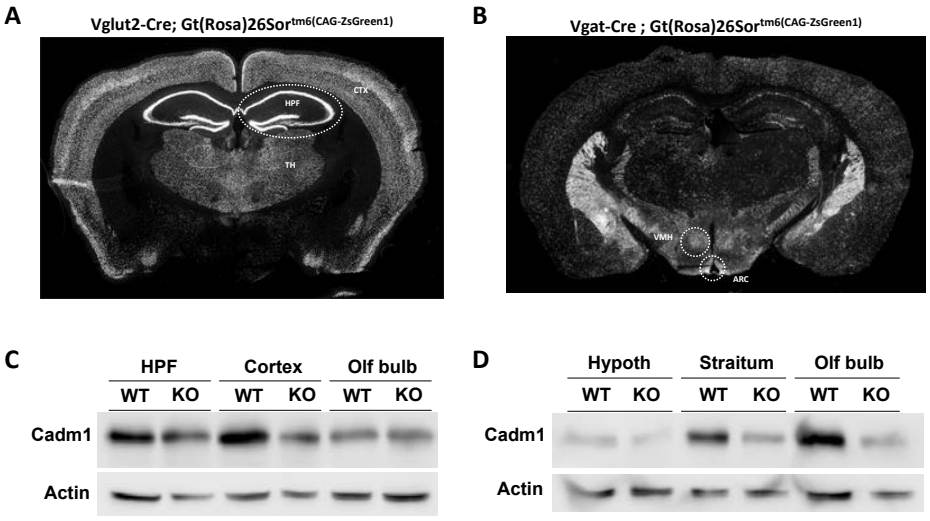

Figure 27: Conditional deletion of Cadm1 in excitatory and inhibitory neurons.
(A and B) Immunofluorescence of coronal sections from Vglut2-Cre of Vgat-Cre mice crossed to the ZsGreen reporter mouse respectively. **(C)** Western Blot analysis of Cadm1 in Hippocampal Formation (HPF), Cortex and Olfactory bulb (Olf bulb) of Cadm1^{Vglut2} mice. **(D)** Western Blot analysis of Cadm1 in Hypothalamus (Hypoth), Striatum and Olfactory bulb (Olf bulb) of Cadm1^{Vgat} mice.

4.4.3 Loss of Cadm1 in Vglut2-positive neurons reduces body weight and improves insulin sensitivity

Interestingly, Cadm1^{Vglut2} exhibit a decrease in body weight, which develops soon after birth and persists throughout adulthood (Figure 28A). Furthermore, blood glucose levels are lower during an insulin challenge compared to wild type littermate controls, suggesting improved insulin sensitivity (Figure 28B). Additionally, hepatic gluconeogenesis was addressed by pyruvate injection, as shown in Figure 28C. In line with improved insulin sensitivity, Cadm1^{Vglut2} show significantly lower glucose level during the pyruvate challenge, and further underlines that loss of Cadm1 in Vglut2-positive neurons improves insulin sensitivity. In order to address the cause of the decreased body weight, mice were characterized in a metabolic cage. Cadm1^{Vglut2} mice are characterized by increased energy expenditure that was normalized by lean body masses. Additionally, Vglut2-Cre mediated deletion of Cadm1 resulted in increased locomotor activity, which at least partially contributed to the increase in energy expenditure (Figure 28D-F).

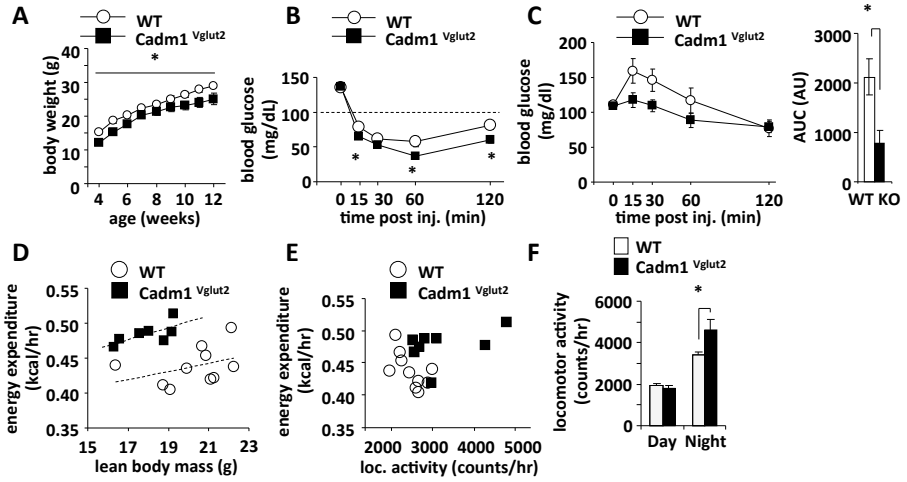

Figure 28: Vglut2-Cre-mediated deletion of Cadm1 reduces body weight and improves insulin sensitivity.
(A) Body weight in Cadm1^{Vglut2} mice and littermate controls from 4-12 weeks of age (n=10-12). **(B)** Glucose measurements during an insulin tolerance test on 12-week old Cadm1^{Vglut2} mice and control littermates (n=5-6). (**C**) Glucose measurements during a pyruvate tolerance test on Cadm1^{Vglut2} mice and littermates and quantification of area under the curve (AUC) (n=6) **(D)** Energy expenditure per individual animals plotted against lean body mass in 12-week old Cadm1^{Vglut2} mice (n=7) and littermates (n=10). **(E)** Energy expenditure per individual animals plotted against locomotor activity in 12-week old Cadm1^{Vglut2} mice (n=8) and littermate controls (n=8). **(F)** Quantification of locomotor activity and food intake measured in 12-week old Cadm1^{Vglut2} mice and littermate controls. (n=7-8).

4.4.4 Cadm1^{Vglut2} mice are protected from diet-induced obesity

In order to test whether loss of Cadm1 in Vglut2-positve neurons protects from diet-induced obesity, Cadm1^{Vglut2} were fed a high fat diet for 10 weeks. As shown in Figure 29A, the decrease in body weight persists during the high fat diet. Furthermore, Vglut2-mediated deletion of Cadm1 also resulted in significantly improved insulin sensitivity in mice receiving a high fat diet for 10 weeks (Figure 29B). Lastly, animals still retain increased energy expenditure and increased locomotor activity (Figure 29C and D). Taken together, loss of Cadm1 in Vglut2-positive neurons protects mice from diet-induced obesity.

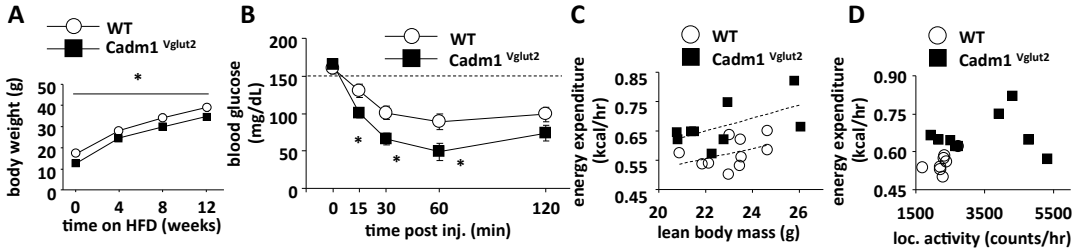

Figure 29: Vglut2-Cre-mediated deletion of Cadm1 protects from diet-induced obesity.
(A) Body weight in Cadm1^{Vglut2} mice and littermate controls from 0-12 weeks on high fat diet (n=10-12). **(B)** Glucose measurements during an insulin tolerance test on 22-week old Cadm1^{Vglut2} mice and control littermates after 10 weeks on high fat diet (n=5-6). **(C)** Energy expenditure per individual animals plotted against lean body mass in 22-week old Cadm1^{Vglut2} mice (n=7) and littermate controls (n=8) after 10 weeks on high fat diet. **(D)** Energy expenditure per individual animals plotted against locomotor activity in 22-week old Cadm1^{Vglut2} mice (n=7) and littermate controls (n=8) after 10 weeks on high fat diet.

4.4.5 Cadm1^{Vglut2} mice are protected from genetic-induced obesity

In addition to the high fat diet as a model for obesity, Cadm1^{Vglut2} mice were crossed to ob/ob mice, a genetic model of obesity. Consistent with the observations observed after feeding a high fat diet, ob/ob Cadm1^{Vglut2} show a significant lower body weight compared to ob/ob mice (Figure 30A). Furthermore, ob/ob Cadm1^{Vglut2} are characterized by significantly lower random blood glucose level, as depicted in Figure 30B. A challenge with insulin resulted in lower glucose level, which suggests improved insulin sensitivity (Figure 30C). Lastly, ob/ob Cadm1^{Vglut2} show dramatically reduced insulin levels compared to ob/ob littermate controls (see Figure 30D). In line with the observations in diet-induced obese mice, loss of Cadm1 in Vglut2 positive neurons protects from diet and genetic-induced obesity.

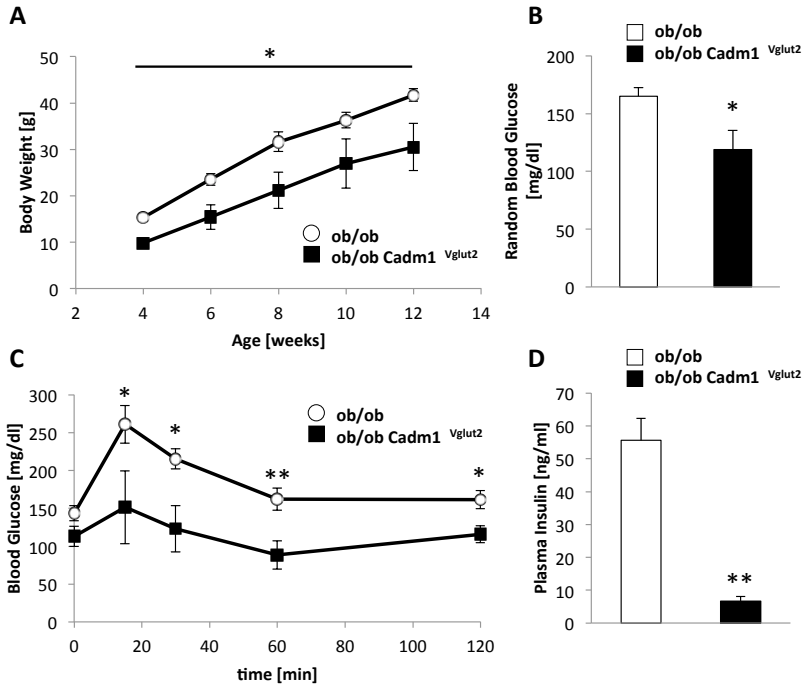

Figure 30: Vglut2-Cre-mediated deletion of Cadm1 protects from genetic-induced obesity.
(A) Body weight of ob/ob and ob/ob Cadm1^{Vglut2} mice from 4-12 weeks of age (n=4-9). **(B)** Random Blood Glucose levels of ob/ob and ob/ob Cadm1^{Vglut2} at 10 weeks of age (n=4-9). **(C)** Glucose measurements during an insulin tolerance test on ob/ob and ob/ob Cadm1^{Vglut2} (n=4-8). **(D)** Plasma insulin levels of ob/ob and ob/ob Cadm1^{Vglut2} at 10 weeks of age (n=4-9).

4.4.6 Loss of Cadm1 in Vglut2 positive neurons results in increased glucose infusion rate

To further verify the improved insulin sensitivity in Cadm1^{Vglut2} mice, hyperinsulinemic-euglycemic clamp studies were performed, which is the gold standard to address whole body insulin sensitivity in rodents. Therefore, plasma insulin levels were constantly raised with an insulin infusion. At the same time glucose infusion was slowly increased until equilibrium, where glucose infusion neutralizes insulin infusion. The clamp study was performed on Cadm1^{Vglut2} mice receiving a high fat diet for 9 weeks. As shown in Figure 31A-C, glucose infusion rate is significantly increased in Cadm1^{Vglut2} mice, further confirming improved insulin sensitivity. An alternative readout is the hepatic glucose output, which reflects hepatic gluconeogenesis. Under basal conditions, Cadm1^{Vglut2} did not show any difference in hepatic glucose output (Figure 31D). However, during the challenge, loss of Cadm1 in Vglut2 positive neurons resulted in significantly decreased hepatic glucose output. Lastly, peripheral glucose uptake is not changed (Figure 31E). In summary, these results further emphasize that

Cadm1^{Vglut2} mice exhibit improved hepatic insulin sensitivity, while glucose uptake in tissues is not altered.

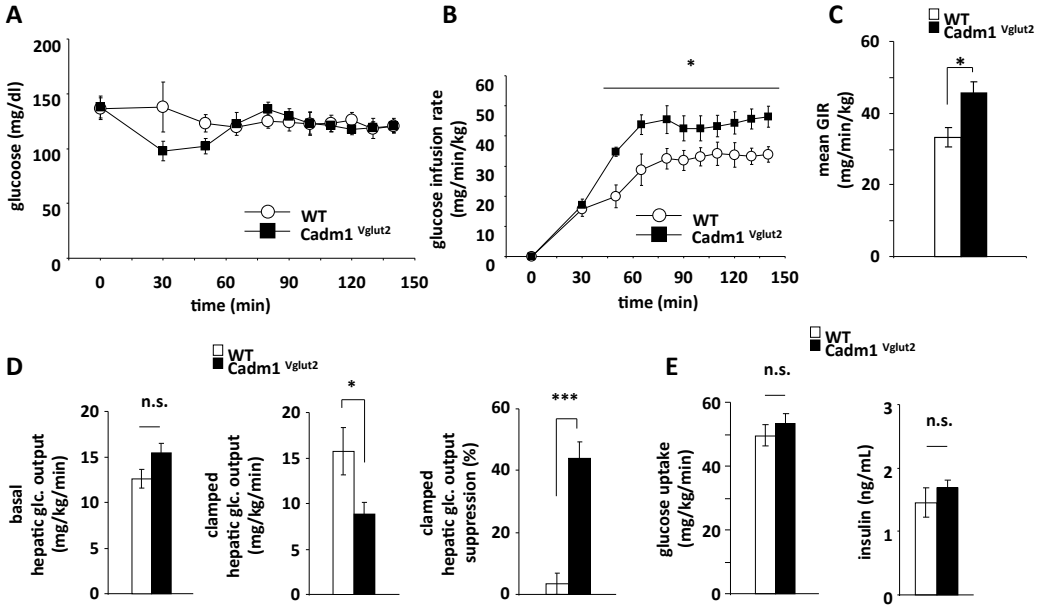

Figure 31: Increased glucose infusion rate observed in Cadm1^{Vglut2} mice during hyperinsulinemic-euglycemic clamp studies.
(A) Plasma glucose concentrations during hyperinsulinemic-euglycemic clamp studies on 12-week old Cadm1^{Vglut2} mice and control littermates (n=4-7). **(B)** Glucose infusion rate during hyperinsulinemic-euglycemic clamp studies on 12-week old Cadm1^{Vglut2} mice and control littermates (n=4-7). **(C)** Mean Glucose Infusion rate during hyperinsulinemic-euglycemic clamp studies on 12-week old Cadm1^{Vglut2} mice and control littermates (n=4-7). **(D)** Endogenous glucose production in the basal and the clamped state in Cadm1^{Vglut2} mice and control littermates (n=4-7). **(E)** Peripheral glucose uptake and plasma insulin levels during the hyperinsulinemic-euglycemic clamp studies (n=4-7).

4.4.7 Loss of Cadm1 in Vgat positive neurons does not contribute to the regulation of energy homeostasis

In order to address if Cadm1 exerts a similar function in other neuronal populations, it was knocked out in inhibitory Vgat-expressing neurons. As shown in Figure 32A, Cadm1^{Vgat} mice did show any significant difference in body weight throughout the studied period. Glucose levels were not significantly changed during a challenge with insulin, but showed a trend to higher levels. Furthermore, the area under the curve (AUC) showed a significant increase in glucose levels leaning towards insulin resistance (Figure 32B). This would suggest that Cadm1 has a counter regulatory function in Vgat-positive neurons. However, Cadm1^{Vgat} mice did not show any significant difference during a challenge with glucose, as shown in Figure 32C. Lastly

loss of Cadm1 in Vgat-positive neurons did not show any difference in energy expenditure, locomotor activity and food intake (Figure 32D-G), further highlighting the function of Cadm1 specifically in Vglut2-positive neurons.

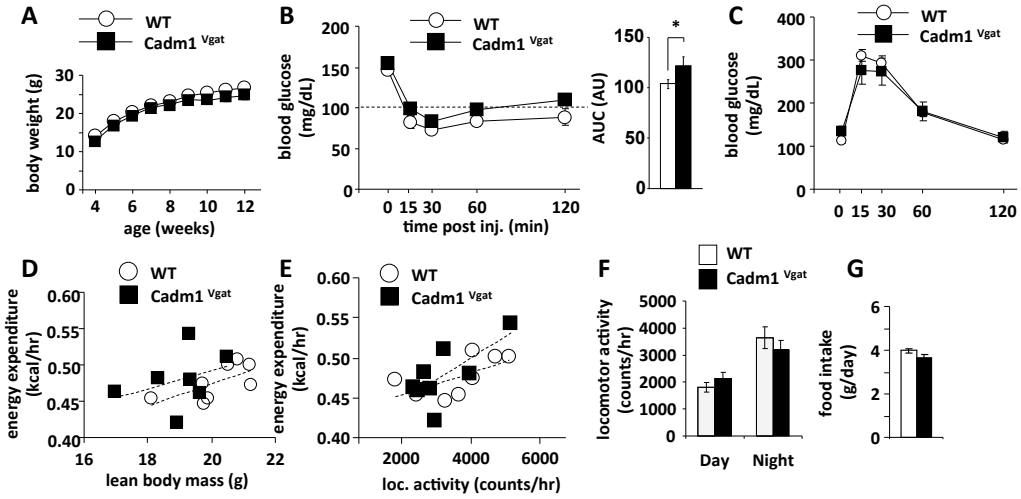

Figure 32: Vgat-Cre-mediated deletion of Cadm1 does not affect body weight and insulin sensitivity.
(A) Body weight in Cadm1^{Vgat} mice and littermate controls from 4-12 weeks of age (n=10-12). **(B)** Glucose measurements during an insulin tolerance test on 12-week old Cadm1^{Vgat} mice and control littermates and quantification of area under the curve (AUC) (n=5-6). **(C)** Glucose measurements during a glucose tolerance test on Cadm1^{Vgat} mice and control littermates (n=6). **(D)** Energy expenditure per individual animals plotted against lean body mass in 12-week old Cadm1^{Vgat} mice (n=7) and littermate controls (n=8). **(E)** Energy expenditure per individual animals plotted against locomotor activity in 12-week old Cadm1^{Vgat} mice (n=7) and littermate controls (n=8). **(F)** Quantification of locomotor activity and food intake measured in 12-week old Cadm1^{Vgat} mice and littermate controls. (n=7-8). (G) Quantification of food intake measured in 12-week old Cadm1^{Vgat} mice and littermate controls. (n=7-8).

4.4.8 Loss of Cadm1 in Vglut2 positive neurons results in altered synaptic composition

Cadm1 was previously been shown to mediate synaptic contacts and loss of genetic deletion resulted in decreased number of excitatory synapses, without affecting the number of inhibitory synapses (Biederer et al., 2002; Robbins et al., 2010). In order to test if conditional deletion of Cadm1 in Vglut2-positive neurons also results in decreased synapse number we acquired electron microscopic (EM) images and quantified the number of excitatory synapses. In contrast to previous studies, we did not observe any change in synapse number (Figure 33A). However, we observed a significant decrease in the length of the post-synaptic density (PSD) (Figure 32B and

C). In order to further confirm this observation, we isolated hippocampal synaptosomes from WT and Cadm1$^{Vglut2\text{-}Cre}$ mice. Western Blot analysis could confirm reduction of Cadm1 expression (Figure 32D). In line with the EM analysis we observed a significant decrease of PSD-95 expression, an important structural component of the post-synaptic density. Taken together, loss of Cadm1 in Vglut2-positive neurons results in reduced PSD length in excitatory synapses without affecting synapse number.

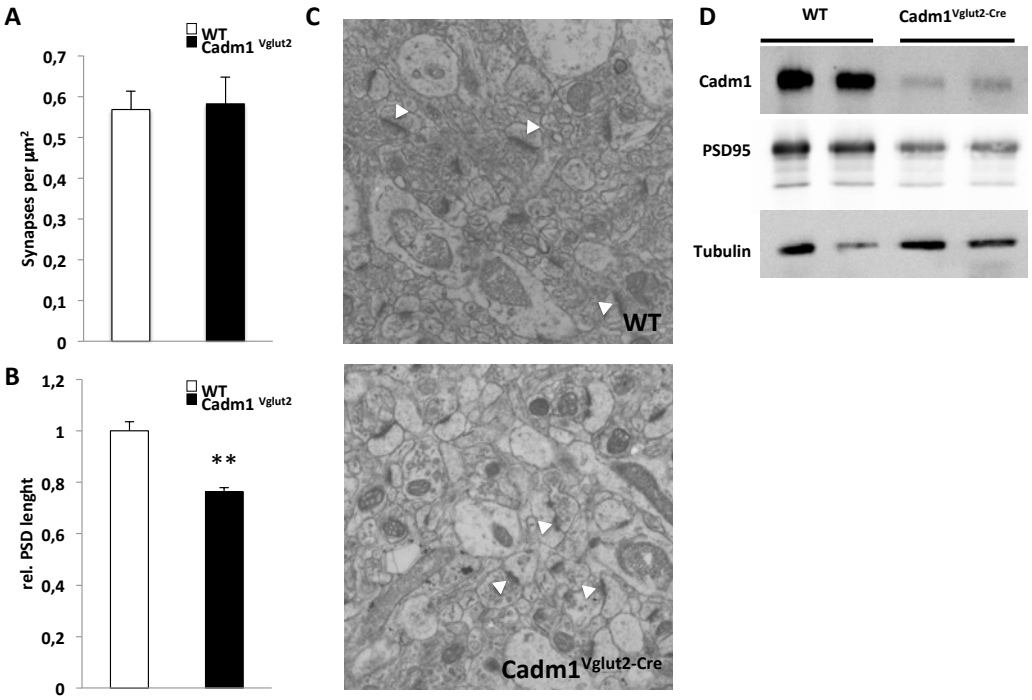

Figure 33: Loss of Cadm1 in Vglut2-positive neurons results in altered synaptic composition.

(A) Quantification of excitatory synapses in hippocampi of Cadm1$^{Vglut2\text{-}Cre}$ mice compared to WT littermate controls (n=4) **(B)** Quantification of post-synaptic densities (PSD) length in hippocampi of Cadm1$^{Vglut2\text{-}Cre}$ mice compared to WT littermate controls (n=4) **(C)** Representative EM images of hippocampi of Cadm1$^{Vglut2\text{-}Cre}$ mice and WT littermate controls. Arrowhead indicates PSD of excitatory synapses. **(D)** Western Blot analysis of isolated synaptosomes from hippocampi of Cadm1$^{Vglut2\text{-}Cre}$ mice compared to WT littermate controls, probed for Cadm1, PSD95 and Tubulin (n=2).

5 Discussion

5.1 Functional insights into the microRNA pathway in the pancreatic islet

In order to maintain normal physiology and organ function under normal as well as stress conditions, a tight control of metabolic homeostasis is crucial (Rottiers and Naar, 2012). Many regulatory pathways have been identified, which contribute to both the pathogenesis and counter regulatory responses to metabolic stress like insulin resistance and hyperglycemia. The miRNA pathway plays an essential role in the post-transcriptional regulation of gene expression and is implicated in cellular processes, including development, stress responses and the maintenance of cellular identity (Bushati and Cohen, 2007; Kaspi et al., 2014; Leung and Sharp, 2010; Mendell and Olson, 2012; Ribeiro et al., 2014a). One of the most abundantly expressed miRNAs in the pancreatic islet is *miR-375* that has been shown to regulate the release of insulin (Poy et al., 2004). Loss of *miR-375* resulted in an increased release of insulin, independently of glucose metabolism and intracellular Ca^{2+} signaling. *MiR-375* targets the ankyrin repeat protein Mtpn, thereby directly affecting insulin exocytosis. Moreover, loss of *miR-375* in mice resulted in decreased beta cell mass and impaired compensatory beta cell hyperplasia in a mouse model of insulin resistance and caused severe hyperglycemia (Poy et al., 2009). MiRNAs are predicted to have up to several hundred targets. Target prediction algorithms such as PicTar and TargetScan predicted around 200 conserved targets of *miR-375* (Friedman et al., 2009; Krek et al., 2005). However, this number is based on computational predictions and relies on seed sequence conservation among species and current models of target recognition (Bartel, 2009). Additionally, efficient suppression of target gene expression by a miRNA, depends also on the stoichiometric ratio of miRNA and target mRNA (Denzler et al., 2014; Guo et al., 2014; Hausser and Zavolan, 2014). Moreover, miRNA and target gene expression can differ dramatically according to developmental stages and changes of the environment (Sokol, 2012; Thornton and Gregory, 2012). Hence, understanding the biological function of miRNAs depends mainly on the experimental validation of targets and their function, both *in vitro* and *in vivo*. Multiple studies have tried to validate some of the predicted targets of *miR-375*, including *Mtpn*, *Pdcd8*, *Rasd1*, *Eef1e1*, *Gphn*, *HuD*, *Cadm1*, *Pdk1*, *Hnf1β* and *Sox9* (El Ouaamari et al., 2008; Poy et

al., 2004; Poy et al., 2009; Wei et al., 2013). However, the functional relevance of these validated targets remains largely unknown.

The miRNA pathway is an important component of cellular stress responses (Leung and Sharp, 2010; Mendell and Olson, 2012). The activity of the pathway can be modulated in different ways in order to maintain the function and the survival of the cell. In the beta cell this includes the adaptation to insulin demand, by the regulation of insulin secretion and cell proliferation. Individual miRNA can be regulated in order to promote or suppress target gene expression. In response to insulin resistance, beta cells silence *miR-184* thereby promoting the expression of one of its targets Ago2 (Tattikota et al., 2014). Moreover, restoring *miR-184* expression during insulin resistance decreased expression of Ago2 and prevented compensatory beta cell expansion. While the molecular mechanisms leading to loss of *miR-184* expression are not completely understood, modulation of insulin sensitivity may be the driving force in the regulation of *miR-184* expression. However, various circulating factors and beta cell autonomous mechanisms might also contribute to these stress responses. In contrast to stress responses, misregulation of several miRNAs has also been implicated in the pathogenesis of various diseases, including metabolic, cardiovascular, malignant and neurodegenerative diseases (Faehnle et al., 2014; Gehrke et al., 2010; Jordan et al., 2011; Kornfeld et al., 2013; Latreille et al., 2014; Png et al., 2012; Trajkovski et al., 2012; Trajkovski et al., 2011). Hence, future studies need to carefully address, whether alterations of the miRNA pathway rather promote a stress response and allow the maintenance of the cellular function or rather drives the progression of the disease. In addition to changes in miRNA expression levels, modulation of the activity of the RISC complex might also contribute to stress responses. Ago2 is a central component of this complex and its activity and stability is modified by posttranslational modifications including phosphorylation (Jee and Lai, 2014; Meister, 2013). Interestingly, Ago2 possesses various phosphorylation sites, which are functionally linked to miRNA loading, subcellular location and miRNA maturation in response to stress (Rudel et al., 2011; Shen et al., 2013; Zeng et al., 2008). It remains to be seen in future experiments, how metabolic stress translates into post-translational modification of Ago2 and how this subsequently modifies the miRNA pathway. Although kinases such as Akt and p38 MAPK are known to phosphorylate Ago2 in other cell types, it has to be further addressed what kinases target Ago2 in the pancreatic beta cell. Kinases are one of the

most interesting targets for drug development and could be used to alter the miRNA pathway in the beta cell (Cohen, 2002).

5.1.1 Argonaute2 regulates beta cell proliferation and the release of insulin

The pancreatic beta cell is highly adaptive and is capable to adjust to changes during metabolic stress including insulin resistance. During such a phase, beta cells proliferate in order to provide more insulin and maintain plasma glucose levels within the normal range (Sachdeva and Stoffers, 2009; Vetere et al., 2014). Increased plasma insulin levels can be achieved either by an increase in the amount of released insulin or by increased beta cell mass. The relative contribution of these two phenomena remains controversial (Araujo et al., 2013; Cavaghan et al., 2000). Hyperglycemia can be either chronic, for instance during insulin resistance and T2D, or transient after ingestion of carbohydrate-rich food. Transient hyperglycemia can be normalized rapidly via glucose stimulated insulin release. However, during chronic hyperglycemia, it has been shown that the beta cells increase their rate of proliferation (Porat et al., 2011). Loss of Ago2 resulted, in accordance with *375KO* mice, in decreased beta cell mass and increased insulin secretion, while transgenic overexpression caused an increase in beta cell mass and decrease in secretion (Poy et al., 2009). Changes in beta cell mass are regulated entirely via proliferative pathways, because apoptosis is not affected in loss and gain of function studies of Ago2. This further underlines the importance of the miRNA pathway in the regulation of beta cell mass. However, in our model an increase in proliferation resulted in decreased insulin secretion. Electron microscopy revealed an increase in beta cell area and volume fraction of insulin containing granules, while the number of docked granules did not change in *βAgo2KO* mice. This may contribute to increased glucose stimulated insulin release and requires remodeling of the cytoskeleton and recruitment of the granules (Seino et al., 2011). Several targets of *miR-375* are linked to these processes, including *Mtpn*, *Rasd1* and *Gphn* (Lellis-Santos et al., 2012; Poy et al., 2004; Suckow et al., 2008). It is not completely understood how Ago2 and *miR-375* regulates this balance between proliferative and secretory pathways. It is feasible that energy stores within the cell are limited. Switching to cell proliferation at the expense of insulin secretion and vice versa is observed in several mouse models (Ohara-Imaizumi et al., 2013). However, this phenomenon requires further experimental evidence. While the majority of the miRNA studies in the beta cell focus on the

secretory and growth pathways, little is known about the energy balance within the cell. The glycolysis and oxidative phosphorylation are crucial to generate substrates like ATP that feed multiple pathways in the cell and might be crucial in the regulation of balance between secretory or proliferative pathways. Hence, establishing the link between the miRNA pathway and mitochondrial function seem to be promising avenue. This can be achieved for instance by using the Seahorse Flux Analyzer or the analysis of the metabolome by mass spectrometry.

5.1.2 Argonaute2 mediates *miR-375* function

Previous studies by the Stoffel group could show that loss of *miR-375* during insulin resistance resulted in impaired beta cell expansion and severe hyperglycemia (Poy et al., 2009). Ago2 is the most abundant member of the Argonaute family of proteins and is a crucial component of the miRNA pathway, mediating the interaction of miRNA with its targets (Tattikota et al., 2013; Wang et al., 2012). In order to study the role of the miRNA pathway in more general, we genetically ablated Ago2 specifically in pancreatic beta cells (*βAgo2KO*) (Tattikota et al., 2014). Loss of Ago2 resulted in improved glucose tolerance, enhanced glucose stimulated insulin release and reduced beta cell mass without affecting insulin sensitivity and body weight. Interestingly, this phenotype reflects the phenotype of the *miR-375* knockout (*375KO*) mouse model and suggests that Ago2 is crucial for *miR-375* function (Poy et al., 2009). In contrast, transgenic overexpression of Ago2 in pancreatic islets resulted in impaired glucose tolerance in spite of increased beta cell mass. In contrast to the *375KO* model, *βAgo2KO* mice did not show any difference in the number of glucagon positive alpha cells. *MiR-375* is expressed in both, alpha and beta cells (Klein et al., 2013; Poy et al., 2004). In contrast to *375KO* mice, where miR-375 is deleted globally, *βAgo2KO* mice perturb miR-375 function specifically in the beta cell. While total loss of *miR-375* in mice resulted in a decreased beta cell mass, it also showed an increase in alpha cell number and circulating glucagon levels. The increase in glucagon may have contributed to mild hyperglycemia, which was not evident in *βAgo2KO* mice. This suggests that *miR-375* exhibits different functions in alpha and beta cells, and excludes the effect of Ago2-*miR-375* axis on the lineage specification during development. However, lineage-tracing studies need to be investigated in order to address this observation more thoroughly. Additionally, it will be of interest what is the function of *miR-375* and the

miRNA pathway in other neuroendocrine tissues, like the adrenal gland and the pituitary. These observations strongly suggest that Ago2 mediates the function of *miR-375*. This is further supported by the observation that loss of Ago2, in both isolated pancreatic islets and MIN6 cells, resulted in up-regulation of *miR-375*-targeted genes. Conversely, over-expression of Ago2 resulted in suppression of *miR-375* targets. In contrast knockdown of Ago1 did not result in any significant change in *miR-375* target gene expression, further underlining the strong functional relation between Ago2 and *miR-375*. This is partially explained by the abundance of the two molecules. Ago2 is the most abundant family member, while *miR-375* is among the highest expressed miRNAs in the pancreatic beta cell. Hence, Ago2 and *miR-375* may constitute one of the most abundant RISC complexes in the beta cell and possesses a strong capacity to regulate target mRNAs. This notion is further supported by immunoprecipitations of Ago2, recovering high amounts of *miR-375* (Tattikota et al., 2013). Lastly, the functional link between *miR-375* and Ago2 is supported by RIP-ChIP experiments and confirms the coexistence of *miR-375*, Ago2 and its targets in one complex. Furthermore these experiments further suggest that modulation of either Ago2 or the miRNA constitutes a potent way to modulate target gene expression in response to environmental changes. Taken together, *miR-375* constitutes one of the most abundant miRNAs in the beta cell and is crucial for the regulation of glucose stimulated insulin release and the regulation of growth. Ago2 mainly mediates suppression of *miR-375*-target genes and modulation of this complex exhibits an important pathway to alter beta cell function and can be achieved by altered *miR-375* expression or adjusting the activity of Ago2 (El Ouaamari et al., 2008; Meister, 2013; Zhu et al., 2013).

5.1.3 Argonaute2 regulates beta cell proliferation in a model of islet hyperplasia

The regulation of beta cell mass is a dynamic process and involves several different pathways (Sachdeva and Stoffers, 2009). The miRNA pathway is known to regulate the compensatory expansion of the pancreatic beta cell mass during insulin resistance and pregnancy (Jacovetti et al., 2012; Latreille et al., 2014; Poy et al., 2009; Tattikota et al., 2014). These responses depend greatly on intrinsic and extrinsic signals and drive proliferation of the beta cell. In order study the role of the miRNA pathway in the regulation of beta cell proliferation, we made use of a well-established tumor mouse model, the RIP-Tag mouse (Christofori and Hanahan, 1994; Hanahan, 1985). This model induces beta cell proliferation due to overexpression of the oncogene large T-

antigen, thereby promoting proliferation and suppression of apoptosis and cell cycle arrest (An et al., 2012). In addition to its well-defined timing of tumor genesis, it also offers several other characteristics, including angiogenesis and the remodeling of the tumor environment (Bergers et al., 2000). Crossing either *375KO* or *βAgo2KO* to this model resulted in decreased beta cell mass, which also involves the rescue of plasma glucose and insulin levels and ultimately prolongs the survival of the animals. Consistent with observations in *375KO* and *βAgo2KO* as well as crosses of these models with insulin-resistant ob/ob mice, *miR-375* and Ago2 a crucial regulators of beta cell mass (Poy et al., 2009; Tattikota et al., 2014). Importantly, these models are characterized by very different mechanisms inducing proliferation. This suggests that the miRNA pathway is a checkpoint in the regulation of beta cell growth, where several growth pathways converge. Islet hyperplasia and tumor formation relies on sufficient vascularization and can limit the proliferative capacity (Brissova et al., 2014; Inoue et al., 2002; Olson et al., 2011; Reinert et al., 2014). Quantification of vascularization in the Ago2KO/RIP mice did show any alteration in the blood vessel area, suggesting that the reduced proliferation is not caused by insufficient blood supply. Another potential mechanism is the cell fate of the pancreatic beta cell. Recent studies suggested that beta cell could lose its identity due to loss of insulin expression and constitutes a cause for beta cell failure during the pathogenesis of T2D (Talchai et al., 2012). This phenomenon is also accompanied by re-expression of transcription factors, which are characteristic for beta cell progenitors. It is also known that RIP-Tag mice show features of dedifferentiation of beta cells, including loss of Insulin, Pdx1, Synaptophysin, Chromogranin A and MafA expression (Hunter et al., 2013). Loss of beta cell identity did result in a more aggressive formation of tumors. We did not observe any obvious differences in our models regarding dedifferentiation and Pdx1 expression (data not shown). Moreover, the tumor burden was reduced in 375RIP animals, suggesting that overall proliferation is affected. However, more precise immunohistological quantification and lineage tracing with a special emphasis on different stages of tumor development is needed in order to address de-differentiation in our model. Lastly, metabolic pathways greatly contribute to the regulation of cell proliferation (Agathocleous and Harris, 2013; Anastasiou et al., 2012; Vander Heiden et al., 2011). The miRNA pathway has also been shown to be a mediator of metabolic pathways within the cell and regulate the flux of nutrients (Eichner et al., 2010; Jiang et

al., 2012). It remains to be seen how the miRNA pathway in the beta cell regulates the consumption and usage of available nutrients, thereby mediating cellular functions such as proliferation, secretion and response to stress.

5.1.4 *MiR-375* targeted genes regulate insulin secretion

Several studies have shown that the miRNA pathway negatively regulates glucose-stimulated release of insulin (GSIS) in beta cells (Latreille et al., 2014; Morita et al., 2013; Plaisance et al., 2006; Poy et al., 2004; Wijesekara et al., 2012). In the current investigation, several *miR-375* targeted genes, including *Gphn*, *Mtpn*, *Ywhaz*, *Rasd1*, *Pdcd8* and *Clock* negatively regulated the release of insulin. This suggests that these target genes potentially mediate insulin secretion upon loss of *miR-375*. While other target genes such as *HuD*, *Rgs16* and *Qk* did not result in any significant difference, loss of *Cadm1* resulted in an increased release of insulin. Since a miRNA can target hundreds of mRNAs, it remains a challenge to identify the true biological targets. Depending on the expression levels and the stability of miRNA:mRNA interactions, it is plausible that some targets respond strongly to miRNA-mediated regulation, while others show only small effects (Hausser and Zavolan, 2014). However, even if the effect on a single target gene is small, a miRNA can target multiple components of a pathway. The synergistic effect in turn can have huge biological relevance and impact biological processes like the GSIS (Small and Olson, 2011). This may also explain why the effect of a single target is rather small and does not reflect loss and gain of function of the miRNA. Indeed, *Mtpn*, *Gphn*, *Rasd1* and *Clock* have been implicated in the regulation of insulin release from pancreatic beta cells (Lellis-Santos et al., 2012; Marcheva et al., 2010; Poy et al., 2004; Sadacca et al., 2011; Suckow et al., 2008). In contrast, not every target gene contributes to the release of insulin. These genes might be involved in other functions of the miRNA, for example in the regulation of growth. Surprisingly, *HuD* did not show a significant impact on secretion, though it was previously shown to regulate the translation of insulin mRNA (Lee et al., 2012). Interestingly, knockdown of *Cadm1* resulted in hyper-secretion of insulin MIN6 cells. This further confirms previous studies of our group, showing that Cadm1 knockout mice hyper-secrete insulin during a glucose challenge. Moreover, Ito and colleagues have shown that loss of *Cadm1* resulted in an increase release of glucagon from the murine alpha cell line αTC6 (Ito et al., 2012). This study could show that loss of Cadm1 results in a decrease of *Connexin36*, an important protein that mediates cell-to-cell

communication. Since Cadm1 is also expressed in beta cells, it might a have similar effect on insulin release, but might also mediate a different function of *miR-375* (Koma et al., 2008; Tattikota et al., 2014). Importantly, one has to take into account, that knockdown of the target genes might also interfere with other cellular processes. For instance an increase or decrease in proliferation might also affect the output of insulin, since the amount of released insulin is normalized by the total insulin content of the cells. Hence, the functional relevance of the individual target genes should also be addressed in more detail in terms of alternative functions. Taken together, *miR-375* targets multiple genes in the beta cell, which synergistically regulate the release of insulin in response to glucose.

5.1.5 *MiR-375* regulate the pancreatic beta cell secretome

The pancreatic beta cell is the sole source of insulin and plays a crucial role in the regulation of glucose homeostasis. Previous studies made use of advanced mass spectrometry approaches to address the protein composition of the beta cell or the insulin containing granules (Hickey et al., 2009; Schvartz et al., 2012a; Schvartz et al., 2012b; Suckale and Solimena, 2010; Waanders et al., 2009). While these studies identified numerous factors that control the assembly and the proper release of the granule proteins, little is known which other factors are co-secreted along with insulin in response to glucose. Using a SILAC based approach we identified about 50 released proteins in response to glucose, termed “secretome signature” that included known granule proteins such as insulin, secretogranin, and chromogranin (Ong et al., 2002; Tattikota et al., 2013). Moreover, we identified several recently described proteins, which are putatively released from the beta cell and modulate its function including NPY, VGF and Nptx1 (Cho and Kim, 2004; Schvartz et al., 2012b; Stephens et al., 2012; Whim, 2011). A substantial number of released proteins are known for their regulatory role in bone physiology, including Gc, Spock2, Bmp1, Fam3c, Igf2 and Pappa2 (Christians et al., 2013; Marini and Blissett, 2013; Nykjaer et al., 1999; Ribeiro et al., 2014b; Wit and Camacho-Hubner, 2011; Zhang et al., 2014). Interestingly, there is a strong link between the pathogenesis of diabetes and bone fragility (Carnevale et al., 2014). This would suggest that beta cell derived proteins regulate bone function and may explain why beta cell dysfunction during T2D results in altered bone physiology. Future studies will help to further study the functional role of co-secreted proteins and

will shed light on the impact on beta cell function or their regulatory role of other organs.

5.1.6 The *miR-375* target *Cadm1* regulates beta cell mass

The cell adhesion molecule Cadm1 has been previously shown to be an important tumor suppressor and regulator of growth (Kuramochi et al., 2001; Murakami, 2005; van der Weyden et al., 2012). Several computational tools predict Cadm1 as a target of *miR-375* that is up regulated after loss of *miR-375* or Ago2 (Poy et al., 2009; Tattikota et al., 2014). Furthermore, we could show that loss of Cadm1 resulted in increased beta cell mass due to increased proliferation. Moreover, deletion of Cadm1 in RIP-Tag mice resulted in decreased blood glucose levels and a trend for a decreased survival rate. This suggests that loss of Cadm1 further accelerated islet hyperplasia in RIP-Tag mice. Compared to other models of tumor genesis such as the p53- and Rb-deficient mice, development of tumors in RIP-Tag mice is very dramatic (Harvey et al., 1995). While this model is suitable to study mechanisms that reduce tumor growth, further increase of proliferation by loss of the tumor suppressor Cadm1 might be difficult to achieve. Hence, future studies should shed more light on this mechanism, by crossing Cadm1KO mice to a less aggressive model of tumor genesis. Lastly, the mechanism how Cadm1 regulates proliferation is not completely understood. However, studies indicate that the short cytoplasmic tail is crucial for this phenomenon and serves as a platform to recruit signaling molecules (Mao et al., 2003). Additionally, it has been suggested that "shedding" is a potential mechanism of Cadm1 action. Here, cleavage of Cadm1 results in the release of c-terminal peptide, which promotes apoptosis (Inoue et al., 2014; Mimae et al., 2014; Nagara et al., 2012). Gene expression analysis in MIN6 cells suggested the PTEN-Akt-Signaling pathway as a mediator of Cadm1 function. Indeed, overexpression of Cadm1 in MIN6 cells resulted in decreased insulin-mediated phosphorylation of Akt. Interestingly, we could also identify secreted proteins from the beta cell after knockdown of Cadm1 using a SILAC-based approach (Tattikota et al., 2013). Moreover, we did observe a substantial overlap between the secretome of RIP-Tag islets and the Cadm1 knockdown secretome in MIN6 cells. Both conditions constitute a proliferative state and hence the secretome signature may shed light on factors that a crucial to mediate proliferation. This involves factors that modulate the extracellular matrix and alter growth and survival pathways in an autocrine fashion. In both models, we did observe up regulation of Igf2, Ambp and Sez6l. These proteins

have been implicated in the regulation of growth and the formation of tumors (Christofori et al., 1995; Huang et al., 2013; Kreiling et al., 2012). In RIP-Tag islets we could also observe down regulation of Igf2r. The Igf2r is a soluble receptor and interferes with the Igf signaling pathway, an important growth and survival pathway that is altered in multiple forms of cancer (Khandekar et al., 2011). Hence decrease the amount of released Igf2r would further promote proliferation. Lastly, the functional characterization of the beta cell secretome, both under normal conditions as well as disease states may shed more light on the function of Cadm1 in the beta cell. Taken together, Cadm1 is a crucial target of *miR-375* and regulates pancreatic beta cell mass. However, it is likely that other targets also contribute to the regulation of cell proliferation and mediate the function of *miR-375* (Figure 34).

We shed light on the functional role of the miRNA pathway in the beta cell and highlight the role of Cadm1 as an important target. In light of the complex cooperation of various tissues in the regulation of glucose homeostasis, it remains to be addressed if Cadm1 also plays a role in metabolism in other organs, particularly in the brain, which constitutes an abundant site of Cadm1 expression.

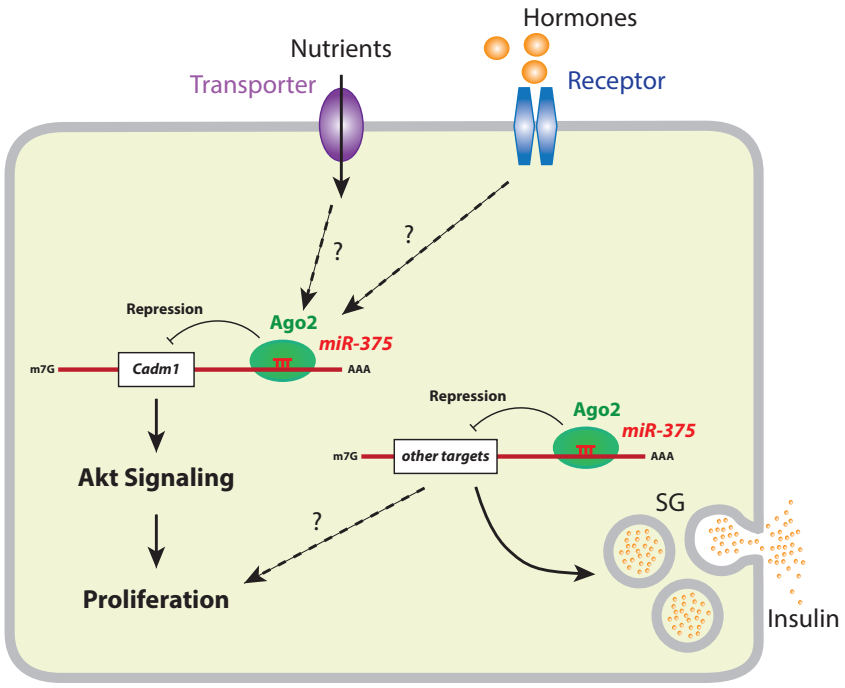

Figure 34: Ago2 regulates the *miR-375* target Cadm1
The activity of the miRNA pathway depends on changes in nutrient availability and circulating hormones. Ago2 mediates *miR-375* function and modulates expression level of Cadm1 and other target mRNAs. Cadm1 mediates the Akt signaling pathway in order to promote proliferation of the pancreatic beta cell. Additionally, Ago2 regulates other miR-375 target genes that in turn regulate beta cell function, including the glucose-stimulated release of insulin and potentially also proliferation.

5.2 Cadm1 as a regulator of energy expenditure and insulin

In addition to its role in the regulation of beta cell function, we also established the functional role of Cadm1 in the central regulation of insulin sensitivity and energy expenditure. Mice deficient in Cadm1 are protected from diet and genetic-induced obesity due to increased locomotor activity, energy expenditure and insulin sensitivity. Moreover, Cadm1 is up regulated in the hippocampus of insulin resistant *ob/ob* mice and its expression can be reversed after restoring insulin sensitivity. These findings underline a significant role of Cadm1 in mediating the physiologic pathways in response to changes in the metabolic state, which determine body mass and glucose homeostasis.

Despite the existence of structurally related immunoglobulin-like adhesion molecules in the brain, Cadm1 seem to be unique in its function, since other family members cannot compensate for its loss. Cadm1 has three other family members (Cadm2-4) that share sequence homology and a similar structure (Biederer, 2006). Moreover, the Cadm family members share similar expression pattern throughout the brain, but also show some more exclusive patterns (Fogel et al., 2007). For instance, Cadm1 is equally expressed throughout the hippocampus, while Cadm2 can be predominantly found in the CA1 region, suggesting that different family members might exert specific functions in a subpopulation of cells. Moreover, the absolute expression levels of individual Cadm proteins might differ and has not been comprehensively addressed yet. Interestingly, Cadm1 expression levels are regulated in the hippocampus according to changes in insulin sensitivity. The administration of a ketogenic diet, which is known to restore insulin sensitivity in ob/ob mice and humans, results in a normalization of Cadm1 expression. This suggests altering Cadm1 levels constitutes an adaptive response to regulate energy homeostasis. Cadm1 is a regulator of synaptogenesis and synapse organization and hence alteration of Cadm1 might contribute to remodeling of neuronal networks and enhance plasticity during stress adaptation (McEwen, 2010; Stranahan and Mattson, 2012).

Since Cadm1 is expressed throughout the brain, it is interesting which subpopulation of cells mediate the effects on energy metabolism or whether different effects might be mediated by distinct cellular populations. Interestingly, $Cadm1^{Vglut2\text{-}cre}$ mice display the same phenotype as mice with a total deletion of Cadm1. This was surprising because the hypothalamus has been considered as the main site in the brain to regulate energy

expenditure and food intake and expresses only low levels of Vglut2-Cre (Vong et al., 2011). The arcuate nucleus in the hypothalamus contains AgRP and POMC neurons, which respond to changes in plasma leptin and insulin levels and fine-tune energy metabolism (Dietrich and Horvath, 2013; Gao and Horvath, 2007). However, the insulin receptor is also abundantly expressed in other region of the brain, including the cortex and the hippocampus (Kleinridders et al., 2014). Similarly, leptin receptors can also be found in the hippocampus (Irving and Harvey, 2014). In addition to the arcuate nucleus, several other regions in the brain have been linked to the regulation of glucose metabolism and energy expenditure, including the hippocampus, the nucleus of the solitary tract and the paraventricular hypothalamus (Kong et al., 2012; Morton et al., 2014; Picard et al., 2014). Vglut2-expressing glutamatergic neurons have been found abundantly in the hippocampus, a site where Cadm1 is abundantly expressed and regulated in response to changes in insulin sensitivity. Moreover, we did not observe any effect on food intake in any of the Cadm1-deficient mouse models and conclude that Cadm1 does not directly affect the function of the hypothalamus (Atasoy et al., 2012; Kong et al., 2012; Sternson et al., 2013). In contrast to $Cadm1^{Vglut2\text{-}cre}$ mice, deletion of Cadm1 in inhibitory neurons ($Cadm1^{Vgat\text{-}cre}$) did not result in changes in food intake, energy expenditure and locomotor activity. Vgat-positive neurons are abundant in the arcuate nucleus of the hypothalamus and have been implicated in the regulation of food intake and energy expenditure. Interestingly, $Cadm1^{Vgat\text{-}cre}$ show reduced insulin sensitivity and might counteract the effect of Cadm1 in Vglut2-positive neurons. Taken together, Cadm1 regulates energy balance according to changes in the metabolic environment in Vglut2-positive neurons in the hippocampus. However, Cadm1 might also have counter regulatory functions in additional neuronal populations.

5.2.1 Cadm1 regulates the organization and function of excitatory synapses

In addition to the family of Cadm proteins, other cell adhesion molecules are also present in synapses, including Neurexin, Neuroligin, N-Cadherin and Neural Cell Adhesion Molecules (Ncam) (Dalva et al., 2007). However these molecules are involved in different synaptic processes, including the structural organization, the regulation of plasticity and the regulation of function, suggesting a non-redundant functional role (Missler et al., 2012). Cadm1 is expressed throughout the brain and is sufficient to form synaptic contacts (Biederer et al., 2002; Fogel et al., 2007). Interestingly, in contrast with previous studies, we did not observe a decrease in the

number of excitatory synapses in the hippocampus of Cadm1$^{Vglut2\text{-}Cre}$ mice (Robbins et al., 2010). This observation is also in line with loss and gain of function studies of isolated hippocampal neurons (Burton et al., 2012). This discrepancy might be explained by the difference in age of the analyzed mice. Synaptogenesis is a dynamic process that peaks during early development and slowly declines during aging (Li et al., 2010). While the Biederer group studied an early time point (4 weeks of age), we picked a later time point in line with the characterization of the mouse (12 to 16 weeks). Interestingly, the difference in body weight of Cadm1$^{Vglut2\text{-}Cre}$ mice appears soon after birth in line with a high rate of synaptogenesis. This might suggest that Cadm1 plays an important role during synaptogenesis in early age, while it might exert an additional function during adulthood. In addition to the mediation of cell-to-cell contacts, Cadm1 might also be involved in downstream signaling. It possesses a short cytoplasmic tail without any known catalytic activity. However, it contains a PDZ as well as a protein 4.1-binding domain (Murakami et al., 2014). Among the known interaction partners are Mupp1, Farb1, Cask, Protein 4.1, Syntenin, Mint1 and Veli. While Cask, Veli and Mint1 mediate the interaction between Cadm1 and the voltage dependent Ca^{2+} channel on the presynaptic side, Mupp1, Farb1, Protein 4.1 and Syntenin organize the active zone on the postsynaptic side (Cheadle and Biederer, 2012; Fujita et al., 2012; Hoy et al., 2009; Samuels et al., 2007). Hence, Cadm1 does not directly mediate any signaling pathways, but rather serves as an important binding partner in the synapse and functions in the maintenance of synaptic protein scaffolds. This is an important feature of the formation of the synapse but also important to transmit information from one cell to the other (Dalva et al., 2007; Missler et al., 2012). Interestingly, we could show a decrease in expression of proteins in the postsynaptic density in Cadm1$^{Vglut2\text{-}Cre}$ mice. In good agreement with the study of Biederer and colleagues, we could also observe a decrease in PSD length in Cadm1 deficient mice, further underlining a functional role of Cadm1 in the organization of the synapse (Fogel et al., 2007; Missler et al., 2012; Ribic et al., 2014; Robbins et al., 2010). Remarkably, we could also observe a regulation of Cadm1 according to changes in insulin sensitivity. This suggests that Cadm1 acts as an important nutrient sensor and modulates insulin sensitivity, energy expenditure and glucose utilization. However, the mechanisms that regulate the expression of Cadm1 in the hippocampus remain to be determined. In addition to peripheral tissues, insulin resistance also affects the brain (Kleinridders et al., 2014). Recently, the miRNA pathway gained attention in the modulation of stress responses in various tissues,

thereby allowing adaptation and the maintenance of the physiologic state (Leung and Sharp, 2010; Mendell and Olson, 2012; Rathjen et al., 2014; Tattikota et al., 2014). The regulation of gene expression in the neurons is not only limited to the cell body, but can be also observed in synapses (Schouten et al., 2013; Schratt, 2009; Siegel et al., 2009). Profiling of small RNAs revealed several miRNAs to be abundant in synaptosomal fractions that are predicted to target Cadm1. Among them are *miR-124*, *miR-128*, *miR-101*, *miR-129*, *miR-148* and *miR-21* (unpublished data). Interestingly, *miR-128* is associated with Ago2 in synaptosomes and has been shown to be a regulator of locomotor activity in mice (Tan et al., 2013; Xu et al., 2013). Moreover, Ago HITS-CLIP analysis of Ago2 in human brain tissue established the interaction of Ago2 with *Cadm1* mRNA (Boudreau et al., 2014). Hence, the miRNA pathway might be involved in the regulation of Cadm1 at the synapse in response to changes in insulin sensitivity. This is further supported by observations in the pancreatic beta cell, where changes in insulin sensitivity modulate the activity of the miRNA pathway and regulate target genes, including Cadm1, in order to adapt to metabolic stress (Tattikota et al., 2014). However, further experimental validation is required to further strengthen the miRNA-mediated regulation of Cadm1 in the brain in response to changes in insulin sensitivity. Taken together, Cadm1 plays a crucial role in the organization of excitatory synapses in the hippocampus and subsequently modulates synaptic transmission in order to regulate insulin sensitivity and glucose utilization (Figure 35).

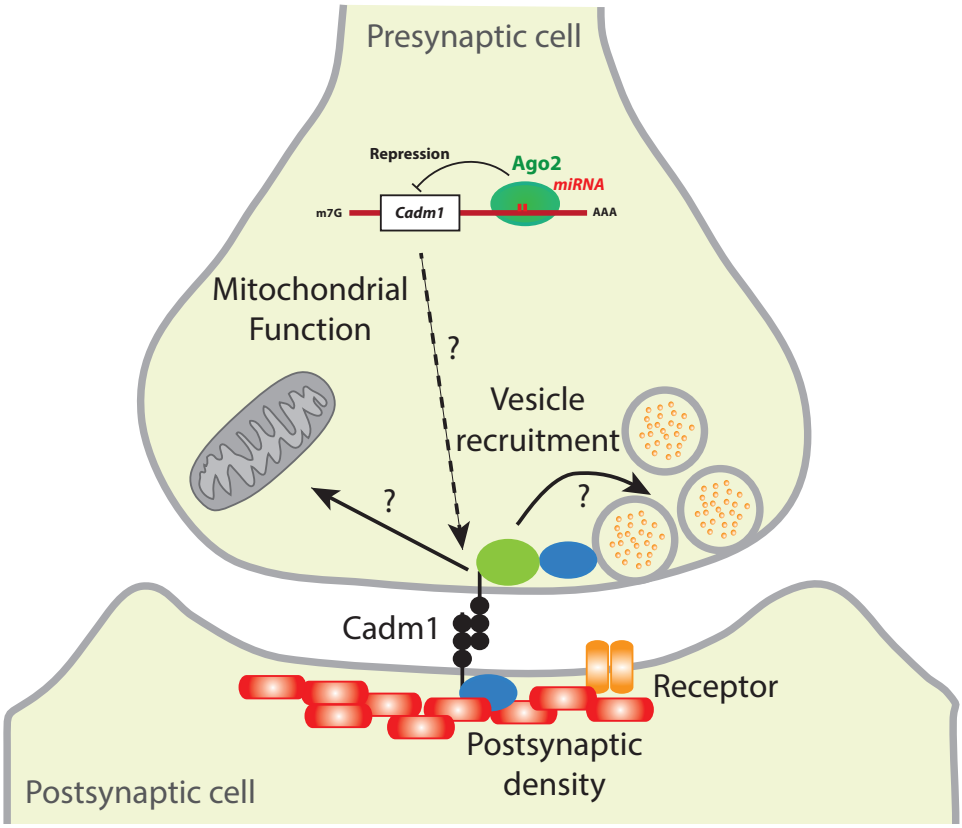

Figure 35: Cadm1 regulates the organization and function of excitatory synapses. Cadm1 mediates trans-synaptic cell adhesion in excitatory synapses, thereby modulating its function. Modulation of Cadm1 expression alters the protein composition of the postsynaptic density. Alternatively, Cadm1 may also regulate the vesicle recruitment in the presynaptic cell and mitochondrial function. Cadm1 expression might be modulated by altered activity of the miRNA pathway in the synaptic compartment. Changes in Cadm1 expression level in the synapse has functional consequences for the synaptic transmission and contributes to energy homeostasis.

6 Summary

MicroRNAs (miRNAs) belong to a class of small non-coding RNAs that are implicated in the post-transcriptional regulation of gene expression. Many insights have been made during the past decade into the regulatory function of the miRNA pathway in health and disease. In the present study, we established Argonaute2 (Ago2), a central mediator of the miRNA pathway, as an important regulator of beta cell function. Loss of Ago2 resulted in decreased proliferation of beta cells and increased glucose-stimulated insulin secretion (GSIS). Furthermore, we could show that Ago2 mediates the function of *miR-375*, a highly abundant miRNA in beta cells, further highlighting the close relationship between these two genes. A small-scale siRNA-based screen revealed that multiple target genes of *miR-375* orchestrate GSIS. Furthermore, using a SILAC based quantitative mass spectrometry approach, we could identify about 50 unique proteins that are co-secreted along with insulin that may have autocrine or paracrine functions. Among the numerous predicted targets of *miR-375*, the cell adhesion molecule 1 (Cadm1) emerged as a suppressor of beta cell proliferation. Interestingly, Cadm1 also exhibits an important regulatory role in the central regulation of glucose homeostasis. Loss of Cadm1 in Vglut2-positive neurons resulted in decreased body weight, increased hepatic insulin sensitivity, increased energy expenditure and protected from diet as well as genetically induced obesity. To our knowledge, we for the first time established a role for a cell adhesion molecule as an important regulator of glucose homeostasis by modulating neuronal circuits and the composition of the active zone within synapses. Furthermore, we could highlight that Cadm1 expression is regulated in response to changes in insulin sensitivity in the hippocampus, emphasizing the adaptive responses of tissues to maintain energy homeostasis.

Future studies will address how changes in circulating hormones and nutrients translate into tissue-specific adaptive responses in gene expression and shed light on the complex network between different cell types in order to maintain energy balance. Lastly, the biological significance of the miRNA pathway in these processes remains to be studied in greater detail that may be crucial for therapeutic intervention.

7 Zusammenfassung

MicroRNAs (miRNAs) gehören zu der Klasse nicht-kodierender RNAs und sind an der post-translationalen Regulation der Genexpression beteiligt. Verschiedene Arbeiten konnten bereits zeigen, dass miRNAs eine wichtige Rolle bei der Aufrechterhaltung der Zellfunktion haben und auch an der Entstehung von Krankheiten beteiligt sind. In der hier vorgelegten Arbeit konnten wir zeigen, dass Argonaute2 (Ago2), ein wichtiger Bestandteil des miRNA Signalweges, an der Regulation des Betazellfunktion beteiligt ist. Der Verlust von Ago2 Expression in Mäusebetazellen resultierte in verringerter Zellproliferation und verstärkter Freisetzung von Insulin nach Stimulation mit Glukose. Darüber hinaus konnten wir zeigen, dass Ago2 die Funktion von *miR-375,* einer stark exprimierten miRNA in Betazellen, vermittelt. Dies verdeutlicht den funktionellen Zusammenhang von *miR-375* und Ago2. Ein Screening von siRNAs in einer Betazellinie hat gezeigt, dass mehrere Zielgene von *miR-375* an der Regulation der Insulinfreisetzung beteiligt sind. Zusätzlich konnten wir mittels Massenspektrometrie 50 Proteine identifizieren, die zusammen mit Insulin in Abhängigkeit von Glukose sezerniert werden und unter Umständen die Funktion von Betazellen und anderen Organen beeinflussen. Das Zelladhesionsmolekül Cadm1 ist ein wichtiges Zielgen von *miR-375* und spielt eine wichtige Rolle bei der Proliferation von Betazellen. Interessanterweise, ist Cadm1 auch ein wichtiger Faktor bei der Regulation der Glukosehomöostase. Der Expressionsverlust von Cadm1 in Vglut2-positiven Neuronen von Mäusen, führt zu einem verringerten Körpergewicht, einem erhöhten Energieumsatz und schützt vor Adipositas. Somit konnten wir erstmalig zeigen, dass ein Zelladhäsionsmolekül ein wichtiger Bestandteil der Glukosehomöostase ist und an der Regulation von neuronalen Netzwerken beteiligt ist. Zusätzlich konnten wir darlegen, dass die Cadm1 Expression in Abhängigkeit von der Insulin-Sensitivität variiert und unterstreicht die adaptiven Eigenschaften von Geweben um den Energiehaushalt aufrecht zu erhalten.

Zukünftige Studien werden an unsere Arbeit anknüpfen und adressieren wie Veränderungen in Hormon- und Nährstoffkonzentrationen zu gewebsspezifischen Anpassungen in der Genexpression führen können. Dabei wird auch der miRNA Signalweg als wichtiger Bestandteil der Regulation und Aufrechterhaltung der Zellfunktion von entscheidender Bedeutung sein und stellt einen vielversprechenden Ansatz zur Behandlung von Krankheiten dar.

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11 Publications

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12 Curriculum Vitae

For reasons of data protection, the curriculum vitae is not published in the electronic version.