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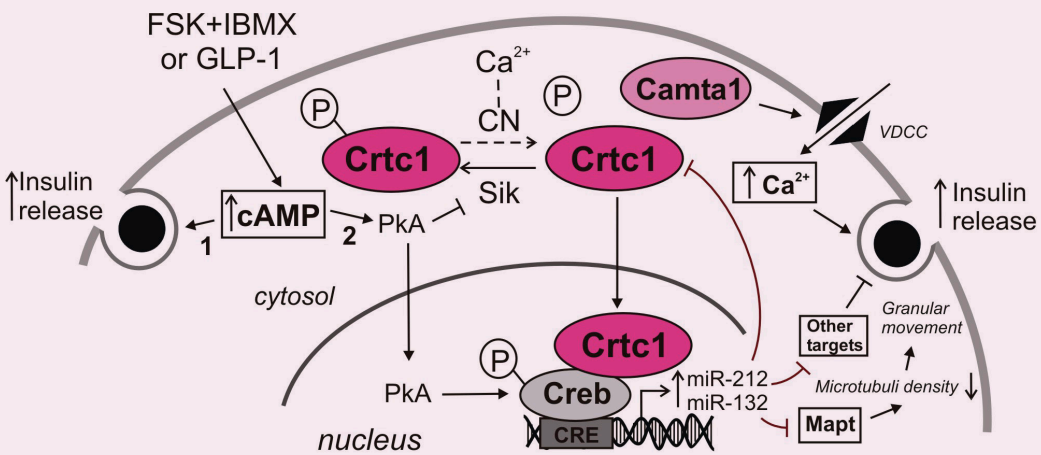

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Abstract

Type 2 diabetes is characterized by hyperglycemia due to insulin resistance in the target tissue and insufficient insulin secretion from the beta cells. Finding new mechanisms and pathways involved in the regulation of insulin secretion from the pancreatic beta cells is of great importance. Our group has earlier found, miRNA-212 and miRNA-132 to be upregulated in the non-obese type 2 diabetic GK rat. We hypothesize that the upregulation of these miRNAs is caused by a misregulation of the presumptive promoter of this miRNA-212/132 cluster. In this thesis I have therefore investigated mechanisms involved in transcriptional regulation of the miRNA-212/132-cluster and their regulation of insulin secretion in the pancreatic beta cell through their target proteins.

The studies were performed in INS1 832/13 cells and primary rat and human pancreatic islets. Cells were incubated at different glucose concentrations in the absence or prescence of cAMP-stimulation using GLP-1 or a combination of forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX). Modulations of expression of different transcriptional factors, miRNA-212 and miRNA-132 were performed using siRNA, LNA and mature miRNA mimics. The rat miRNA-212/132 promoter was cloned into a luciferase construct to evaluate the activity of the miRNA-212/132 promoter at different conditions, a DuoLink experiment was performed to elucidate interactions between different proteins and an Ago2-RNA-immunoprecipitation was performed to verify a direct gene target of miRNA-132. Results were evaulated by qPCR, Western blot, immunofluorescene, glucose stimulated insulin secretion, Ca^{2+} measurements and the patch clamp technique.

In paper I, AMPK-related protein kinases Sik1-3 and co-transcriptional factor Crtc1 were shown to regulate miRNA-212 and miRNA-132 expression in pancreatic beta cells, possibly through Creb/Atf-1. Moreover, Crtc1 and miRNA-212/132 regulated insulin secretion. The effect of Crtc1 on insulin secretion was found to be only partly via miRNA-212/132. Also, the expression of SIK1 correlated positively with miRNA-132 levels in human pancreatic islets.In paper II, Camta1, a novel regulator of insulin secretion in pancreatic beta cells, was shown to regulate Ca^{2+}-influx. Further Camta1 influenced miRNA-212/132 expression and interacted with the T2D implicated transcriptional factor Nkx2.2 at non-physiological glucose levels. In paper III, Mapt (tau) was proven to be a conserved direct target of miRNA-132 in beta cells. Overexpression of miRNA-132 was suggested to modulate the microtubule network and earlier stages of the insulin secretion process through Mapt and putative targets Sox6 and Isl1.

In this thesis I report Crtc1, Camta1, miRNA-212 and miRNA-132 to be regulators of insulin secretion in insulin secreting beta cells, and that miRNA-212 and miRNA-132 are regulated by cAMP and Ca^{2+}, through Crtc1, Camta1 and Creb/Atf1. While Crtc1 is suggested to generally increase insulin secretion, Camta1 more likely increases the Ca^{2+}-influx of the beta cell and miRNA-212/132 regulate insulin secretion through translocation of insulin granules (e.g. Mapt) and insulin transcription (e.g. Sox6 and Isl1).

In this thesis I have shown that miRNA-212 and miRNA-132 are likely to have an important function in the insulin secreting beta cells.This work will help us to a better understanding of how changes in the beta cell lead to the pathogenesis of T2D and hopefully the identified regulating molecules may contribute to the development of new therapeutic drugs.

Key words: T2D, miRNA, GLP-1, cAMP, SIK, CRTC1, CAMTA1, transcriptional regulation, MAPT(tau), beta cell, insulin secretion, SOX6, ISL1

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Till min familj

Content

List of papers included in this thesis

The thesis is a summary of the following papers, which in the text will be referred to by their roman numerals.

I. Helena A Malm, Ines G Mollet, Christine Berggreen, Marju Orho-Melander, Jonathan LS Esguerra, Olga Göransson, Lena Eliasson (2016) Transcriptional regulation of the miR-212/miR-132 cluster in insulin-secreting β-cells by cAMP-regulated transcriptional Co-activator 1 and Salt-Inducible Kinases. *Molecular and Cellular Endocrinology*, 2016 Jan 13. pii: S0303-7207(16)30010-7. Doi: 10.1016/j.mce.2016.01.010

II. Ines G Mollet, Helena A Malm, Anna Wendt, Marju Orho-Melander, Lena Eliasson (2016) Integrator of Stress responses Calmodulin Binding Transcription Activator 1 (Camta1) Regulates miR-212/miR-132 Expression and Insulin Secretion. *J Biol Chem*, 2016 Jul 8. pii: jbc. M116.716860. doi: dx.doi.org/10.1074/jbc.M116.716860

III. Helena A Malm, Ines G Mollet, Jonathan LS Esguerra, Marju Orho-Melander, Lena Eliasson (2016) Microtubuli associated protein tau (Mapt) is a conserved and valid target gene of miRNA-132 regulating early stages of inuslin secretion in beta cells. *Manuscript*

Papers not included in the thesis

- Vishal A Salunkhe, Ines G Mollet, Jones K Ofori, Helena A Malm, Jonathan LS Esguerra, Thomas M Reinbothe, Karin G Stenkula, A Wendt, Lena Eliasson, Jenny Vikman (2016). Dual Effect of Rosuvastatin on Glucose Homeostasis Through Improved Insulin Sensitivity and Reduced Insulin Secretion. *EBioMedicine,* 2016 Jul 9. pii:S2352-3964(16)30312-7. doi:10. 1016/j.ebiom. 2016.07.007

- Jones K Ofori[a], Helena A Malm[a] , Ines G Mollet, Lena Eliasson, Jonathan LS Esguerra (2016). Minimal influence of cell density on the expression of miRNA-375 and its targets in rat and human insulin-secreting cell lines. *Manuscript*

Abbreviations

AC	Adenylate cyclase
ADP	Adenosine diphosphate
ADRA2A	Alpha-2A adrenergic receptor
AGO2	Argonaute-2
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATF-1	Activating transcription factor 1
ATP	Adenosine triphosphate
CaMK	Calmodulin kinase
cAMP	Cyclic AMP
CAMTA	Calmodulin activated transcriptional activator
CART	Cocaine and Amphetamine regulated transcript
CBP	CREB binding protein
CDK5	Cyclin-dependent-like kinase 5
cDNA	Complementary DNA
CG-1/CM2	(A/C/G)CGCG(C/G/T)
CGRP	Calcitonin gene-related peptide
CN	Calcineurin
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
CREM	cAMP-responsive element modulator
CRTC	cAMP-regulated transcriptional co-activators
ECL	Enhanced chemiluminescence
ELISA	Enzyme linked immunosorbent assay
EXODIAB	Excellence of diabetes research in Sweden
FSK	Forskolin
FW	Forward
GABA	Gamma aminobutyric acid
GIP	Glucose dependent insulinotropic polypeptide
GK-rat	Goto-Kakizaki rat
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
GSK3beta	Glycogen synthase kinase 3 beta
GWAS	Genome Wide Association study
HNF-1	Hepatocyte nuclear factor 1
HRP	Horse radish peroxidase
HRPT1	Hypoxanthine-guanine phosphoribosyltransferase
IAPP	Islet amyloid polypeptide

IBMX	3-isobutyl-1-methylxanthine
ICER	Inducible cAMP early repressor
INS1, INS2	Insulin gene
iRNA	RNA interference
ISL1	Insulin gene enhancer protein
K_{ATP} -channel	ATP- sensitive potassium channel
LKB1	Liver kinase B1
LNA	Lock nucleic acid
LUDC	Lund University Diabetes Center
MAFA	V-Maf Avian musculoaponeurotic fibrosarcoma oncogene homolog A
MAPK/ERK	Mitogen activated protein kinase
MAPT	Microtubuli associated protein tau
MARK/PAR1	Microtubuli-affinity regulating kinases
miRNA	MicroRNA
mRNA	Messenger RNA
MSK1	Mitogen- and stress-activated protein kinase
MYOD	Myogenic differentiation 1
ND	Non diabetic
NEUROD1	Neuronal differentiation 1
NFAT	Nuclear factor of activated T-cells
NFT	Neurofibrillary tangles
NKE	5´-NAAGTG-3´
NKX2.2	NK2-homeobox 2
p90RSK	Ribosomal s6 kinase p90 or MAPK-activated protein kinases1
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAX4	Paired box protein 4
PCR	Polymerase chain reaction
PDX1	Pancreatic and duodendal homeobox 1
PKA	Protein kinase A
PKC	Protein kinase C
PP-cells	Pancreatic polypeptide cells
PPIA	Peptidylprolyl isomerase A
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PTBP2	Polypyrimidine tract binding protein 2
PVDF	Polyvinylidene difluoride
REV	Reverse
RIA	Radioimmunoassay
RIP	Rat insulin promoter
RIP	RNA-immunoprecipitation
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid

RT-qPCR	Reverse transcriptional quantitative real-time PCR
SCL2A2	Glucose transporter gene 2
SIK	Salt inducible kinase
siRNA	Small interfering RNA
SNAP-25	Synaptosomal-associated protein-25
SNARE	Soluble N-ethylmaleimidesensitive factor Attachment Protein receptor
SNP	Single nucleotide polymorphism
SOX6	SRY-box 6
SRP	Signal recognition particle
SYT	Synaptotagmin
T2D	Type 2 diabetes
TCF7L2	Transcription factor 7-like 2
TGN	Trans golgi network
TKII	Tau kinase II
UTR	Untranslated region
VAMP-2	Vesicle associated membrane protein-2
VDCC	Voltage-dependent Ca^{2+} -channels
WHO	World Health Organization
ZNT08	Zink transporter 8

Introduction

MicroRNAs

In 1993 Victor Ambrose and colleagues reported the identification of small non coding RNAs in the nematode *C. elegans* [1]. Later studies confirmed the presence of the small non coding RNA, now referred to as microRNA, with the function to repress the expression of proteins [2].

MicroRNAs (miRNAs) are small, 20-22 nucleotides long, non-coding RNAs that downregulate mRNA expressions of coding genes [3]. In this negative post-transcriptional regulation the miRNAs bind to specific motifs in the 3'UTRs of mRNAs in a complementary manner [4]. In the genome, many of these small RNAs are located in introns of protein coding genes, others are found in intergenic regions. Pri-miRNAs are double-stranded hairpin precursors which are subsequently cut by the enzyme Drosha to create pre-miRNAs (Figure 1). Such pre-miRNAs are then further processed to produce mature, active, miRNAs by the enzyme Dicer. A RNA-induced silencing complex (RISC) is then formed to inhibit target mRNA transcription. The RISC complex consists of the Argonaute 2 (AGO2) protein, which bind the mature miRNA and enables binding between the miRNA and a mRNA target. This results in either mRNA degradation or translational repression and give reduced expression of the target protein/proteins [3].

Until today, over 2500 human mature miRNAs have been annotated [5]. One miRNA can inhibit the expression of one single gene target or many gene targets, and the expression of a single gene target can be inhibited by one or several different miRNAs [6-9]. During the last few years altered miRNA expression has indeed been indicated in various pathophysiological states like cancer, cardiac hypertrophy, neurodegeneration, autoimmunity and also diabetes mellitus [10-17]. MiRNAs have been shown to be differentially expressed in pancreatic islets of the non-obese type 2 diabetic GK-rat [18]. MiRNAs can be enriched in specific tissues where they control specific events [19]. This suggest the existence of networks of more or less tissue-specific miRNAs working together in fine-tuning the intrinsic activities of a particular event or pathway [20]. Indeed miRNAs have been

suggested to function as rheostats, optimizing the expression of their surrounding genes in negative feed-back loops [19].

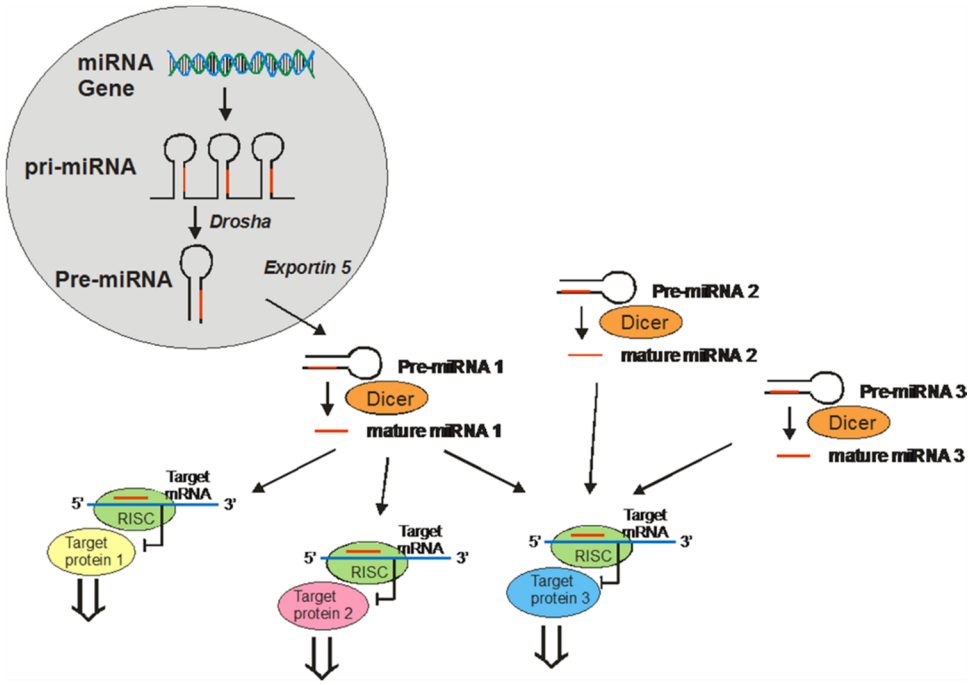

Figure 1
Biogenesis of miRNAs (courtesty of L. Eliasson).

Diabetes mellitus

Diabetes mellitus affects at least 9% of the adult population in the world (2014) and the majority of patients with diabetes have type 2 diabetes (T2D) [21]. T2D is characterized by hyperglycemia due to insulin resistance in the target tissue and insufficient insulin secretion from the beta cells. Hyperglycemia develops when the beta cells fail to secrete enough insulin [22]. Insulin is released from the pancreatic beta cells in a biphasic manner and a characteristic for T2D is a loss of first phase insulin secretion together with decreased secretion in the second phase [23-26].

T2D is a multifactorial, polygenic disease, that is influenced by both *environmental* (such as age [27], physical activity, diet, obesity [28], intrauterine environment [29]) and *genetic* [30-33] factors. Genome wide association studies (GWAS) have identified several SNP-variants that associate with reduced insulin

secretion, insulin resistance and/or T2D [33, 34]. Also, recently it has been proposed that epigenetic changes, as a result of the environment, induce T2D [27, 35-37] Epigenetic changes introduce changes in the gene function that do not alter the nucleotide sequence and this include e.g. DNA methylation and histone modifications.

The first miRNA that was reported to be implicated in insulin secretion was the islet abundant miRNA-375, that was demonstrated to reduce insulin secretion through its target myotrophin [38]. Shortly after, miRNA-9 was discovered as a repressor of insulin secretion and exocytosis. This miRNA was shown to target the transcription factor Onecut-2 regulating the expression of granuphilin, an inhibitor of beta cell exocytosis [39]. After these discoveries several other miRNA have been shown to regulate insulin secretion. These miRNAs will be mentioned more in detail when introducing the insulin secretion process [17, 40]. The importance of miRNAs in beta cell function has been highlighted in work on different mice models of beta cell specific Dicer knockout (induced from birth under the RIP promoter or induced in adulthood by tamoxifen) [41-43]. These mice did not have the ability to create mature miRNAs in beta cells and suffered from progressive impairment of insulin secretion and glucose homeostasis, i.e. they developed diabetes.

Pancreatic islets

The pancreas consists of an exocrine and an endocrine part. The exocrine pancreas is responsible for production and secretion of digestive enzymes that are important for the chemical decomposition of food components and nutrients in the duodenum. The endocrine part is responsible for the secretion of hormones mainly involved in the regulation of blood glucose levels. It consists of cell aggregates spread through the exocrine pancreas called islet of Langerhans (Figure 2). The pancreatic islets were first described in 1869 by the German pathologist Paul Langerhans and were therefore later named “The islets of Langerhans” [44]. The human pancreas has approximately 1 million islets. However, the endocrine part of the pancreas still only comprises 1 % of the total mass of the pancreas.

The majority of the cells within the islets constitute insulin secreting beta cells, and the human islets consist to 60-80% of beta cells [45, 46]. The beta cells were first described in the guinea pig 1907 when Lane and colleagues noticed that some cells in the islets were stained basophilic, b=beta [47]. It has been estimated from ultra-structural analyses that a beta cell contains more than 10 000 insulin granules. Of these, ~600 granules are docked (attached to the plasma membrane),

and ~200 of the docked granules are primed and ready to be fused with the plasma membrane [23].

During the 1960s Grimelius et al. first distinguished alpha cells from the other cell types in the human islet [48]. The alpha cells secrete glucagon and populate the human islets up to 40%. In 1931 Bloom et al. were first to report the somatostatin secreting delta cells in human, which constitute 5-10 % of the islet [49]. Pancreatic polypeptide cells (PP cells, gamma cells) that constitute 2-20% of the islet were discovered in human islets 1975 by Larsson and colleagues [50] and finally, the Ghrelin cells (epsilon cells) that at most populate the islets to 10% were discovered as late as 2002 by Wierup and colleagues.[51].

Human islets contain fewer beta cells and more alpha cells than mouse islets [52]. The beta cells are dispersed fairly equal in the human islets while in mouse islets the beta cells are situated predominately at the surface [46].

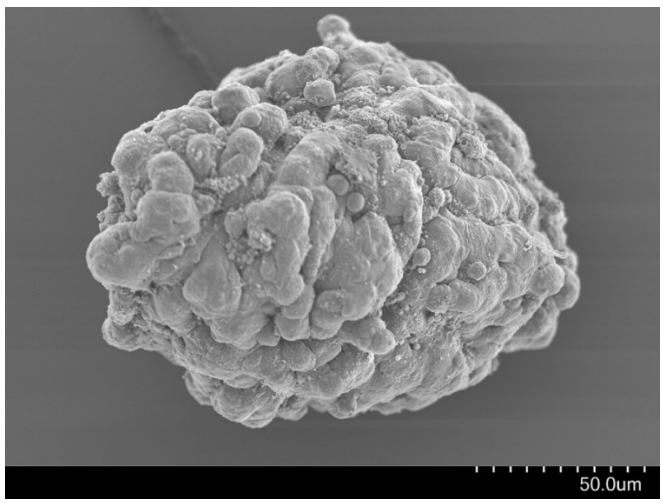

Figure 2
A scanning electron microscopy (SEM) image of a human pancreatic islet of Langerhans (courtesty of L. Eliasson)

Hormonal secretion

Insulin

Banting and Best were first to isolate insulin (= insula, island) in 1921 [53] and the discovery revolutionized the treatment of patients with diabetes at the time. Insulin is released from the pancreatic beta cells when blood glucose levels are high. The hormone facilitates the uptake of glucose in liver, muscle and adipose tissue, stimulates energy storage in liver and adipose tissue and represses gluconeogenesis and lipolysis.

Under normal conditions insulin release from the beta cell is biphasic upon a rapid and constant stimulation by glucose [24, 25]. The first phase is rapid, lasting for 5-10 min and it has been estimated that about 40-80 insulin granules per cell are released during this phase. The second phase of insulin release is longer with a plateau after approximately 2-3h [26, 54]. On a cellular level the biphasic insulin secretion has been associated with different groups of release compatible insulin-containing granules prior to exocytosis [25, 55-57].

Other islet hormones

Glucagon is released from the alpha cells when blood glucose levels are low. This hormone stimulates the hepatic glucose production which results in elevated blood glucose levels. Opposite to insulin, glucagon promotes gluconeogenesis in the liver and lipolysis in cells [58]. Somatostatin is released from the delta cells and reduces insulin and glucagon secretion in paracrine manner [59]. The pancreatic polypeptide, secreted from the PP cells, is involved in inhibition of insulin- and somatostatin secretion [60, 61]. Little is known about the function of ghrelin, that is secreted by the epsilon cells, however this hormone has been suggested to have an inhibitory effect on insulin secretion [62].

The beta cell does not only secrete insulin, but also e.g. GABA and islet amyloid polypeptide (IAPP) [63, 64]. GABA is believed to repress glucagon secretion from the alpha cells [65-67]. IAPP is thought to have an autocrine function to inhibit insulin secretion. IAPP is also suggested to be responsible for the amyloid plaques that can be observed in islets from T2D patients [68].

Incretins and the islets

As a response to elevated glucose levels in the gut after a meal, the gastrointestinal hormones glucagon-like peptide 1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) are released into ileum and duodenum, respectively. These hormones, named incretins, enhance glucose stimulated insulin secretion and are responsible for a large elevation of insulin secretion after a meal. This is called the incretin effect. This effect has been shown in experiments, were the orally administrated glucose stimulates insulin secretion much more than if the glucose is given intravenously [69-71].

Both incretins have been demonstrated to increase beta cell proliferation and reduce beta cell apoptosis and to stimulate insulin and somatostatin secretion. GLP-1 inhibits glucagon secretion, whereas GIP stimulates the secretion from alpha cells. [69, 70, 72] .

Incretins promote release of insulin from the beta cell. The binding of incretins GLP-1 or GIP to the G-protein coupled receptors leads to increased levels of cAMP within the cell [60, 70]. Experimentally the diterpene forskolin (FSK), an

adenylate cyclase (AC) activator, and IBMX, a phosphodiesterase inhibitor, can be used to elevate endogenous cAMP levels in the cell [73, 74].

Neuronal regulation of insulin secretion

Data from rodent islets and cell lines show that beta cells are innervated and under the influence of transmittors in the parasympathetic nervous system (i.e. acetylcholine, vasointestinal peptide (VIP), PACAP [75] and CART) for the *stimulation* of insulin secretion. Transmittors in the sympathetic nervous system (i.e. noradrenaline, NPY and galanin [76]) and the sensory nervous system (CGRP, substance P, CART) inhibit insulin secretion [77]. The presence of nerve terminals inside the human pancreatic islets has been questioned. However it is clear that the beta cells have muscarine and adrenergic receptors and recent findings highlights the alpha-2A adrenergic receptor (ADRA2A). A polymorphism in, or nearby, this gene is associated with increased expression of ADRA2A and reduced insulin secretion [78]. Treating patients, homozygous for this polymorphism, with an ADRA2A antagonist improves their insulin secretion [79]. Other studies suggest that acetylcholine is secreted from the alpha cells and thereby regulate insulin secretion through a paracrine mechanism [80].

Regulation of insulin secretion release

Stimulus secretion coupling

Glucose is the main stimulator of insulin secretion from beta cells (Figure 3) and is taken up into the cell through a glucose transporter (GLUT); in rodents mainly through GLUT2 and in humans through GLUT1 and 2 [81, 82]. Inside the cell, glucose is converted to glucose-6-phosphate with the help of glucokinase (GK). GK has a high threshold for glucose phosphorylation and therefore destines glucose for glycolysis only when extracellular glucose levels are high. Glucose-6-phosphate can then enter the mitochondria resulting in generation of ATP at the cost of ADP. Beta cell metabolism of glucose is different from other cells. E.g. GK is the only hexokinase expressed in beta cells. This hexokinase only allow glucose to enter glycolysis when extracellular glucose is high. Moreover, the monocarboxylate transporter (MCT1), that transport lactate and pyruvate, is not present or expressed at low levels in the beta cells and therefore termed disallowed [83]. This is important since it allows only glucose and not pyruvate to be metabolized by the beta cell. Interestingly, it has been shown that MCT1 in beta cells is a target gene of miRNA-29 [84]. Moreover, several other disallowed beta cell genes are targets of miRNAs [41].

ATP and the decreasing ADP concentration will close the ATP-dependent K^+ -channel (K_{ATP} channel). The flow through this channel maintains the cell

membrane at a negative membrane potential. When closed, the membrane potential will depolarize from -70 mV to more positive potentials. In mouse beta cells the closing of the channel results in depolarization of the membrane and the opening of voltage-sensitive L-type Ca^{2+} -channels. The influx of Ca^{2+} through L-type Ca^{2+} -channels triggers exocytosis of insulin granlues [85]. In the human beta cell the signal transduction works slightly different. Here the closure of the K_{ATP} -channel leads to depolarization of the plasma membrane and opening of voltage-dependent T-type Ca^{2+} -channels and voltage-dependent Na^{+} -channels which will further depolarize the membrane to open L-type and P/Q-type Ca^{2+} -channels. Influx of Ca^{2+} through P/Q-type Ca^{2+} -channels triggers exocytosis. [22, 86] (Figure 3). It has been demonstrated that K_{ATP} and voltage-dependent Ca^{2+}-channels (VDCC) are activated by cAMP [87]. In the matter of miRNAs and the beta cell, ion channels involved in beta cell stimulus secretion coupling, subunits of the voltage-dependent Na^{+} -channels, have been shown to be targets of miRNA-375 [88].

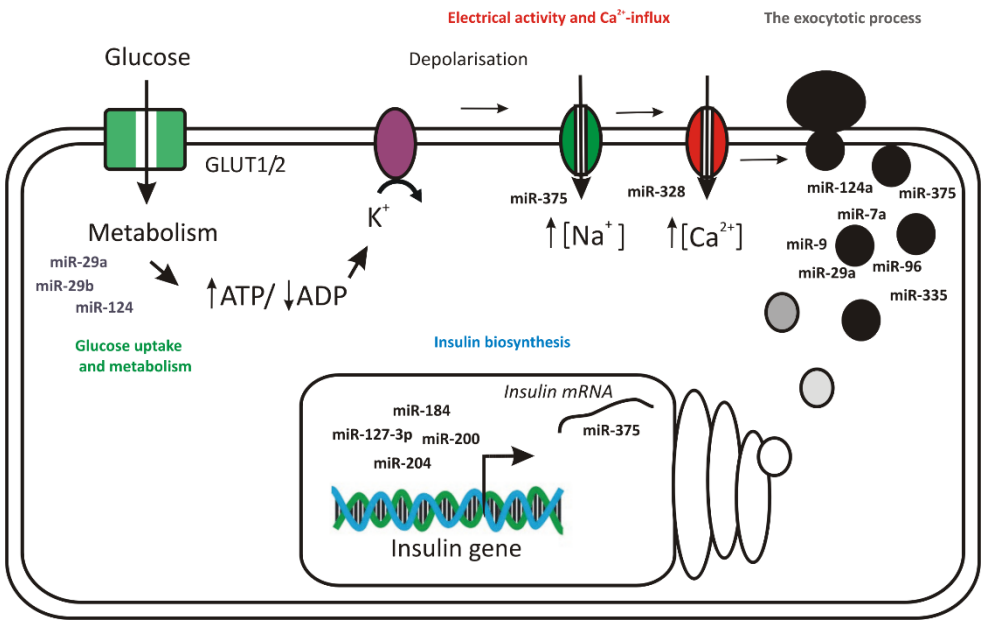

Figure 3.
Stimulus secretion coupling and insulin biosynthesis. The involvement of miRNAs (modified from Eliasson et al. 2014)

The exocytotic machinery

The exocytotic process in the beta cell includes 1) recruitment of granules through transport and mobilization of granules to the plasma membrane, 2) docking of granules to the plasma membrane, 3) priming to make the granules release

competent and finally 4) Ca^{2+} dependent fusion of insulin containing granules with the plasma membrane.

Involved in these processes are several proteins including Munc-18, granuphilin, synaptotagmins (SYTs) and the SNARE-proteins. The SNARE-complex consists of three proteins, SNAP-25, syntaxin1 and synaptobrevin/VAMP-2. The exocytotic proteins facilitate the exocytotic process by bringing the granules closer to the plasma membrane for fusion to occur [54, 86, 89, 90].

The granules undergo the process called priming once docked at the plasma membrane. Priming is an ATP, cAMP, Ca^{2+} and temperature-dependent process [91-93]. Cyclic AMP has been shown to act both in a PKA-dependent and PKA-independent way directly on the exocytotic process of insulin granules in the beta cell. The latter involves the interaction with cAMP-GEFII and increases priming [91, 92, 94].

During the docking and priming process, the plasma membrane proteins Syntaxin 1 and SNAP-25, pair with the granular protein synaptobrevin/VAMP-2. The formation of the SNARE-complex enables fusion of the insulin granules. The trigger of fusion is a local increase in intracellular Ca^{2+} concentration [85] , which is sensed by the Ca^{2+} sensitive protein synaptotagmin. Interestingly, islets from T2D donors have reduced expression of syntaxin 1, munc-18 and several of the synaptotagmins [95]. Munc-18 has been shown to be a direct target of miRNA-335, suggesting a function of this miRNA in the control of the exocytotic machinery. Moreover, both miRNA-375 and miRNA-9 have been shown to effect exocytotic response in beta cells through their targets. Syntaxin 1 has been demonstrated to be a target to miRNA-29a. Thus, miRNAs have a central role in the regulation of insulin granule exocytosis [17, 40, 96].

The level of Ca^{2+} rises within the cell due to increased Ca^{2+} influx through the Ca^{2+} channels, which leads to increased exocytosis [85].

Mobilization of insulin granules

The cytoskeletal involvement in the regulation of insulin secretion from the pancreatic beta cell has been vastly investigated over the years [97, 98] but is not yet fully elucidated. However, last years improved technics in electrophysiology and microscopy have allowed detailed investigation of this process.

The microtubule network in the pancreatic beta cell consists of a complex non-directional network [99]. Granules are *transported* by directional granular movement along the microtubules from the Golgi towards the plasma membrane. This directional movement is orchestrated by ATP-demanding motor proteins kinesin and dynein [99-101]. Oscillatory random diffusion is a pre-requisite for directed granule *transport* (microtubule-guided salutatory jumps), and it is

generally believed that this diffusion or non-directional movement is also important for the delivery, *recruitment* of the granules to the plasma membrane [102].

The cortical actin web, acts as a negative regulator of insulin secretion at the plasma membrane in close interaction with protein myosin 5a and other exocytotic proteins [54, 90, 103]. Myosin 5a guides the insulin granule through the dense actin network and is believed to be involved in both the first [103] and the second phase [104] of insulin secretion. It is still debated though, whether directional transport or the random movements are the most important for insulin granule transport [105] and whether cytoskeletal involvement is required for the amplification of glucose stimulated insulin secretion [106, 107]. In addition to glucose, cyclic AMP amplifies insulin secretion from the beta cell [22, 92], however this process seems to be independent of actin filaments [108]. Lately, also microtubule has been reported to negatively regulate insulin secretion and still a lot remains to be explored about the crosstalk between microtubule and actin filaments [109, 110].

Transcriptional regulation of genes involved in insulin secretion

Insulin biosynthesis

The promoter of the gene encoding insulin has several motifs to which different transcription factors bind [111]. The most studied transcription factor that regulate expression of the insulin gene is the Pancreatic and duodendal homeobox 1 (PDX1) [112, 113]. Other transcription factors that have motifs on the insulin promoter are ISL1[114], MAFA [115] , NEUROD1, MYOD, HNF-1, PAX4 and PAX6 [111]. Recently TCF7L2, a transcription factor in the Wnt-signaling pathway, has been shown to be a master regulator of insulin production and processing in the pancreatic beta cell. TCF7L2 regulates ISL1 that controls proinsulin production and processing through, among others PDX1, MAFA, NKX6.1 and ZNT08 [116]. A number of cyclic AMP responsive elements (CRE)-sites have been identified [60] on the insulin gene promoter. Members of the CREB/ATF-family are known to bind to these CRE-sites (see below). Incretins and glucose induce insulin gene transcription and insulin biosynthesis through the Ca^{2+}/calcineurin /NFAT pathway [117, 118] or mediate by PDX1 [69]. MiRNAs are involved in the regulation of insulin gene transcription, e.g. MAFA is a direct target of miRNA-204 [119]. (Figure 3)

Insulin biosynthesis includes several steps [111, 120] . The insulin gene encodes for a 110 amino acid long precursor, preproinsulin. The preproinsulin mRNA is translated to the preproinsulin protein at the ribosomes on the endoplasmatic reticulum (ER). For this precursor protein to be guided by the signal recognition

particles (SRP) into the lumen of the ER it contains a hydrophobic N-terminal signal peptide. The proinsulin is further guided into the *trans*-Golgi network (TGN)[120]. Immature secretory granules coated with clathrin molecules then buds out from the TGN. During the maturation of the insulin granules, the interior of the granule is acidified (for the proinsulin convertases to work), the Zn-proinsulin hexamers are formed, and the proinsulin is cleaved into an α- and β-chain joined by disulfide bridges [120, 121]. The C-peptide is cleaved off and is secreted together with insulin.

cAMP responsive element-binding protein (CREB)

Cyclic AMP responsive element (CRE) -binding sites are found within the promoter region of many genes [122, 123]. The ubiquitous transcription factor CREB binds to these CRE-sites [60]. As mention above, the insulin gene promoter has several CRE-sites [124]. CREB and its family members are basic region leucine zipper (bZIP) proteins and forms dimers when binding to the CRE-motif on the DNA [125]. Among the CREB/ATF family of transcription factors are Activating Transcription Factor 1 (ATF-1) and CRE-Modulator (CREM). A truncated form of CREM, ICER, has been shown to be a repressor of CREB-regulated genes [126, 127]. CREB can be activated by cAMP through protein kinase A (PKA) mediated phosphorylation at Ser-133 [128] (Figure 4). Besides cAMP, also elevated levels of Ca^{2+} and growth factors activate CREB trough MAPK/ERK- and Akt-pathways. That is, besides PKA, many kinds of kinases have CREB as their substrate such as MSK1, AKT, MAPK, $p90^{RSK}$, PKC, CaMKII and CaMKIV [128-131]

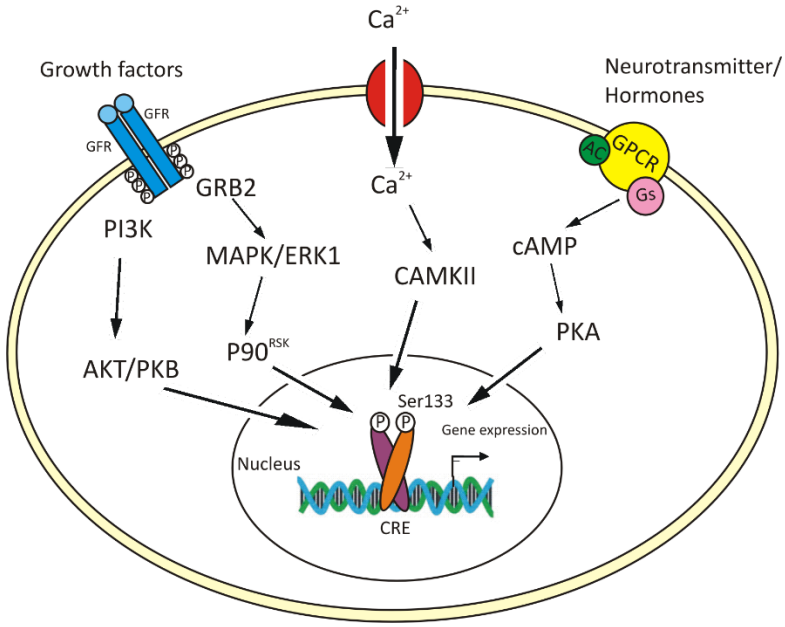

Figure 4
Several signaling pathways stimulate CREB (Courtesty of H. Malm)

Co-activation of CREB

Several co-transcriptional factors work with CREB; CREB binding protein (CBP), p300 and the cyclic AMP-regulated transcriptional co-activators (CRTCs) [60].

CBP and p300 are functional homologs and interacts with CREB at the phosphorylated Ser-133 [132-135]. These coactivators of CREB are histone acetylase transferases [136, 137] and help recruit RNA polymerase II to the promoter [129]. CBP can be phosphorylated by CaMKIV [138]. CBP has been shown to increase pancreatic beta cell proliferation [139] and to be implicated in diabetic nephropathy through TGF-β1 [140].

CRTCs are, as CREB, sensors for hormonal and metabolic signals [60]. The CRTCs exist as three different isoforms; CRTC1, CRTC2 and CRTC3 [141, 142]. Under basal conditions in the cell, the phosphorylated (two serine residues) CRTC protein is kept in the cytoplasm outside the nucleus, by the 14-3-3 protein [60] [143]. Cyclic AMP and Ca^{2+} promote CRTC entrance into the nucleus. Studies in neurons claim that Ca^{2+} -influx enable the nuclear translocation of CRTC1, while cAMP is important for controlling that CRTC1 remains in the nucleus [144].

The Ser/Thr phosphatase calcineurin (CN) dephosphorylates CRTC upon Ca^{2+} stimulation, releasing the co-activator from the 14-3-3 proteins. This allows CRTC

to enter the nucleus and join CREB/ATF-1 at the promoter. The phosphorylation of CREB/ATF-1 by cAMP and PKA is independent of CRTC [135].

The CRTC protein is phosphorylated, i.e. inhibited, by Salt Inducible Kinases (SIKs) [145, 146], which in turn are inhibited by cAMP induced PKA [147]. Salt inducible kinases (SIKs) exist in three isoforms and belong to a large family of AMP-activated kinase related kinases [148, 149]. The serine/threonine protein kinase, liver kinase B1 (LKB1), is a master regulator of 13 AMPK-related kinases [148, 150], including the SIKs.

SIK2 and CRTC2 have been implicated in Ca^{2+} and cAMP mediated glucose sensing through CREB in pancreatic beta cells as well as the regulation of gluconeogenesis in liver, where CBP/p300 have a roll in the stimulation of gluconeogenesis [143, 151]. Further CRTC2 is required for beta cell proliferation and insulin secretion [152].

Calmodulin binding transcription activators (CAMTAs)

CAMTAs are transcription and co-transcription factors that are highly conserved in eukaryotes [153, 154] and were first discovered in plants in stress responses [155, 156]. CAMTA exists in two isoforms in vertebrates: CAMTA1 and CAMTA2 and they recognize CM2 or CG-1 motifs on the DNA of promoters [157], [156]. CAMTA1 has a risk allele for T2D and is a known target of miRNA-9 which has been implicated in the regulation of insulin secretion, and miRNA-17-5p, that has been implicated in neuronal cancer cells [158-161]. CAMTAs have been shown to interact with NK2 transcription factors, e.g. NKX2.5, and to oppose/compete with class 2 histone deacetylases (HDACs) in cardiac muscle in mice [157]. NKX2.2 has been associated with diabetes [162, 163]. NK2-related factors are known to bind to NKE elements in the promoter region of the genes [164].

Aims

In this thesis I aimed to investigate mechanisms involved in transcriptional regulation of the miRNA-212/miRNA-132 cluster and their subsequent regulation of insulin secretion in the pancreatic beta cell trough their target proteins.

Specific aims

I. Investigate cAMP-mediated transcriptional regulation of the miRNA-212/miRNA-132 cluster and involvement of further upstream proteins in insulin secreting beta cells.

II. Perform a bioinformatic analysis of the promoter of the miRNA-212/miRNA-132 cluster and investigate the possibility of regulation of miRNA-212/miRNA-132 expression and insulin secretion by calmodulin-binding transcription activators (Camtas) and transcription factor NKX2.2 in insulin secreting beta cells.

III. Validate a novel gene target of miRNA-212/miRNA-132 and investigate the potential role of this target in the regulation of beta cell insulin secretion.

Material and methods

Cells and tissues

Pancreatic beta cell lines

The rat insulinoma beta cell line clone, INS1 832/13 is derived from a secreting INS1 clone and contains a stable transfection with an additional *human* proinsulin gene. The cells respond to glucose and other secretagogues, and both K^+_{ATP} - channel dependent and K^+_{ATP} - channel independent signal transduction pathways can be activated [165]. The cell line has lately often been utilized for electrophysiological studies of insulin secretion [166]. All these aspects render this cell line suitable as a beta cell model system. One should keep in mind that INS1 832/13 cells are insulinoma cells that differ in some aspects from pancreatic islets [167]. However, these cells are suitable for, e.g. signal transduction analysis studies and transfection studies followed by the monitoring of insulin secretion and ultrastructural changes. In my thesis I used these cells in all three papers when studying signal cAMP- and Ca^{2+} -mediated signal transduction pathways that regulate miRNA-212 and miRNA-132 expression. I also used these cells to study the modulating effect that miRNA-212 and miRNA-132 have on their targets in mRNA and protein expression studies.

Animal models in diabetes

The Goto-Kakizaki (GK) rat is one of the best characterized animal models of spontaneous T2D, being a non-obese diabetic inbreed from Wistar rat [168]. The model has reduced islet beta cell mass and reduced glucose stimulated insulin secretion, which reflect the complex pathogenesis behind T2D [168]. In my thesis we used pancreatic islets and single pancreatic beta cells from GK and Wistar rats in paper I and II. In paper I we used islets to confirm findings in the cell line and and in paper II we used DNA from these rats when cloning the promoter of miRNA- 212/132 cluster.

Human islets

In my thesis we used data obtained in human islets from cadaver donors. In paper I we used data on gene expression, HbA1c and T2D status. In paper II we used human islets to confirm our findings in rat pancreatic islets and INS1 832/13 cells. Human islets were derived from the Human Tissue laboratory (HTL) at Lund University diabetes centre (LUDC) and the strategic network EXODIAB through a collaboration with the Nordic Network of islet transplantation.

Modulation of gene expression

For a transient overexpression of a miRNA, mature miRNAs were transfected into the cell (Applied Biosystems, USA) using lipofectamine. For downregulation of miRNAs specific locked nucleic acids (LNAs) were designed and transfected into the cell [169]. LNAs are nucleic acids analogs where the carbohydrate is locked with a methylene bridge (Exiqon, Denmark).

For a transient silencing of a gene, predesigned small interfering RNAs (siRNAs) were transfected into the cell [170, 171]. The processing of siRNAs from the nucleic genome is similar to that of miRNAs. However unlike miRNAs, siRNA is introduced as double stranded RNA (dsRNA). The siRNA is fully complementary and the siRNA targeted mRNA is cleaved at the RISC complex resulting in reduced expression of the specific gene [172-174].

In my thesis we used these techniques in paper I-III to modulate miRNA-212 and miRNA-132 expression, and to silence specific proteins in order to investigate their impact on a specific signaling pathway or on general beta cell function.

Quantification of mRNA and miRNA expression

In the experiments where quantified RNA expression was measured, the single stranded nucleic acid was converted to the stable complementary DNA (cDNA). Messenger RNAs (mRNAs) were converted to cDNAs by reverse transcriptase, using random primers that align randomly to the different RNA strands. Specific Taqman® primers then amplified the cDNA of the gene of interest. Since the amount of material, from which the RNA was extracted in the first place may differ slightly, as well as the loading of the sample, endogenous controls were used for normalization of the samples. *Hrpt1* and *Ppia* were used as endogenous controls. The C_t-values of the target genes and the endogenous controls were

normalized to each other using GeNorm [175] and the different samples were analyzed together using the ΔΔCt-method [176].

MiRNAs are structurally situated as loops in the genome, and transcribed into small RNA loops (pri-miRNAs) before maturing. Therefore specific stem loop primers were used when converting miRNAs and other small RNAs into cDNA loops. The endogenous controls used for normalization were ubiquitous small RNAs (e.g. *U87* and *U6*) that were converted into cDNA loops by specific stem loop primers. The cDNA loops of target miRNAs and endogenous controls were amplified and incorporated with specific Taqman® probes, normalized to endogenous controls and the different samples were analyzed using the ΔΔCt-method [175, 176].

In my thesis we used qPCR in papers I-III to monitor the modulation of miRNA-212 and miRNA-132 expression as well as the mRNA expression of genes situated upstream or downstream of miRNA-212 and miRNA-132.

Seamless cloning®

In Seamless cloning®, sequence specific primers are used to introduce new ends to the vector and the insert to create overlapping sequences to the ends of the vector and insert. This facilitates site directed recombination by sequence homology. Using seamless cloning, the insert is fast and highly efficiently directed to the ends of the vector and recombined into place. Furthermore, multiple inserts can be introduced simultaneously (Thermo Fisher Scientific, USA). In my thesis, I used Seamless cloning in paper II when cloning a 2.6kb fragment of the promoter region of the miRNA cluster miRNA-212/miRNA-132 into a Metridia Luciferase vector. The following primers were used, FW primer: 5´ATCTCGAGGAGAA-CCTGCTTCACATCATAGG-3´and REV primer: 5´-ATGAATTCACTCCTG-ATCCCATCAGTTCACCA-3´.

Luciferase assay

Luciferase assays are used to elucidate the functional impact of transcription factors on regulation of promoter activity (or other regulatory sequences). *Metridia longa,* a marine copepod (shrimp), secretes the luminescent protein, *luciferase,* and the gene encoding luciferase is used as a reporter gene in the vector constructs containing the regulatory sequence of interest (Takara Bio, USA). Luminescence is recorded by an UV-spectrophotometer and the quantity of luciferase expression indicates the activity of transcription factors on the investigated regulatory sequence of interest [177]. In my thesis we used luciferase assay in paper II, when

monitoring the activity of the promoter of the miRNA-212 and miRNA-132 under various conditions when silencing *Camta1* in INS1 832/13 cells.

Western blot

In my thesis I used Western blot in paper I and III for the detection, quantification (e.g. degree of phosphorylation, dephosphorylation) and identification of Crtc, Sik proteins and Mapt (tau) proteins after various treatments. The protein lysate sample was heated up to near boiling for a few minutes for the denaturation of the proteins. The sample, with unfolded proteins, was then loaded on a SDS-polyacrylamide gel and subjected to an electrophoresis where proteins in the sample were separated by size. The proteins were further transferred (blotted) onto a nitro-cellulose or PVDF membrane and introduced to a primary antibody against the specific protein of interest for identification. For detection, a secondary horseradish peroxidase (HRP)-linked antibody against the source of the primary antibody was linked to this antibody. HRP catalyzes the reaction where luminol is (eventually) oxidized to 3-aminophthalate. The light signal (428 nm), that is emitted in the process, was enhanced by certain chemicals and read by an enhanced chemiluminescence (ECL)-reader. (Abcam, UK). The quantification of the signal was performed in the software of the ECL-reader. For proper quantification, a ubiquitous endogenous protein for the particular tissue needs to be detected as a loading control. The cytoskeletal protein beta-actin cyclophilin b (Ppib) was used for our experiments. Ppib is an ubiquitous protein folding catalyst in the cell [178, 179].

Ago2-RNA immunoprecipitation

In my thesis I used Ago2-RNA-immunoprecipitation in paper III for the validation of *Mapt* as a direct target of miRNA-132 in INS1 832/13 cells. MiRNA targets are by tradition validated by a dual luciferase assay [180, 181] (see above). However, the Ago2-RNA-immunoprecipitation has recently emerged as a new technique based on immunoprecipitation of Ago2, followed by the retrieval of attached RNA. The advantages of this technique compared to luciferase assay are the possibility of validating several targets of the miRNA in the same experiment and the monitoring of the miRNA binding to *endogenous* mRNA. As mentioned in the *Introduction*, miRNAs associate with the Ago2 protein and bind (mostly) to the 3'UTR of the target mRNA. By immunoprecipitation of Ago2 in a particular cell line using a specific primary antibody conjugated to beads, it was possible to retrieve the mRNA and miRNA attached to the protein (and to each other). Construction of cDNA and qPCR of the Ago2 attached RNA-material was used to

identify these products. Occasionally the qPCR reads may give heterodimers of a miRNA and its target mRNA, unintentionally validating the miRNA target [182]. The validation was, conducted by overexpressing the miRNA and yet receiving an enrichment of target mRNA compared to control, since a high amount of miRNA would tether more target mRNA to the Ago2 protein. qPCR primers were constructed for the miRNA target site on the 3´UTR of the target mRNA for amplification of a sequence not more than 20 bps flanking the miRNA target site (Sigma-Aldrich, USA). To ensure that the accumulation of target mRNA to the Ago2 protein was not unspecific the cell line lysate was also presented to an unspecific primary antibody. I used the following in-house designed qPCR primers for the 3´UTR of the *Mapt* mRNA.: FW primer: 5´-CTGTGAATGTCCATATAGTGTACTG-3´ and REV primer 5´-ACAGCAACA-GTCAGTGTAAATC-3´, negative control *Pdha1*: FW primer: 5´-CGAACAA-GGGTCTTTCTGTGTA-3´ and REV primer: 5´-CACACACAAATCCTGCG-TTTAC-3´ (IDT, Integrated DNA technologies, USA)

Immunofluorescent visualization using confocal microscopy

The principle of confocal microscopy is based on usage of a pinhole, which leads to that only the light that orginates from the focal plane is captured and the total image gets sharp. By changing the focal plane, several sharp images can be captured which enables visualization of details in each image, and build up of 3D-images if desired [183].

In fluorescent confocal microscopy several different fluorophores excited by different lasers and emitting light at different wavelengths can be used simultaneously. The biomolecule that is to be visualized is targeted with a specific primary antibody, and the secondary (or the primary) antibody is labeled with a fluorophore. In my thesis I used this technique in paper I when investigating the nuclear translocation of the transcription factor Crtc1 in INS1 832/13 cells and pancreatic beta cells. In paper II we used this technique to confirm silencing of *Camta1*. In paper III I used this technique to investigate the effect on Mapt (tau) and alfa-tubulin distribution and intensity when overexpressing miRNA-132 and when silencing *Mapt*. All imaging experiments were analyzed with a laser scanning confocal microscope (Zeiss LSM-510) with Argon 488nm and 2-photon lasers. Images were analyzed using Zen software (Zeiss, Germany).

A *Proximity ligation assay* can be used, when aiming to identify a true interaction at a proximity of 40 nm between co-localized proteins. This is a technique where the two proteins, presumed to interact, are targeted with specific antibodies made in different species. These antibodies are in turn targeted with secondary antibodies labeled with + and - ends that when closed will hybridize with a

"substrate" that in turn will form a circle. The circle is amplified and labeled with a fluorescent dye which provides a signal in the case of interaction (Olink, Sweden). In my thesis we used this technique in paper II when investigating the possible interaction between transcription factors Camta1 and Nkx2.2.

Hormone secretion assays

In paper I-III in my thesis, insulin secretion and insulin content in primary rat islets or insulin-secreting cells were measured using radioimmunoassay (RIA) or enzyme linked immunosorbent assay (ELISA).

Statistical analysis

In my thesis data are generally presented as the mean ± standard error of the mean (SEM) or in the case of Western blot experiments as the mean±standard deviation of the mean (STDEV). All p-values reported are two-sided and unpaired. For experiments with more than two variables the two-way ANOVA was used. For multiple comparisons a Tukey post hoc-test or a Holm-Bolmferroni test was used. Graphpad prism was used for the statistical analysis of all experiments.

Results

Paper I

Our group has previously found miRNA-212 and miRNA-132 to be upregulated in the GK rat compared to the Wistar rat [18]. Since we also found these miRNAs to be specifically regulated by glucose [18], we decided to investigate the transcriptional regulation of miRNA-212 and miRNA-132.

MiRNA-212 and miRNA-132 are modulators of insulin secretion and are induced by cAMP in pancreatic beta cells

We initially showed miRNA-212 to positively regulate insulin secretion in beta cells. We were next interested in how miRNA-212 and miRNA-132 expressions are regulated. Incretins are known to increase intracellular cAMP and thereby enhance insulin secretion [70]. Since there are known conserved CRE-sites on the presumptive promoter of miRNA-212 and miRNA-132 in mouse brain [184, 185] (Figure 6.), we investigated whether glucose, with or without the second messenger cAMP, would stimulate miRNA-212 and miRNA-132 expression in pancreatic beta cells. We confirmed a short-term glucose regulation in INS1 832/13 cells and reported a rapid and strong long lasting expression after cAMP-incubation in pancreatic beta cells. Incubation with the incretin GLP-1 also elevated miRNA-212 and miRNA-132 expression.

Crtc1 regulates insulin secretion and expression of miRNA-212 and miRNA-132 in INS1 832/13 cells

Expressions of miRNA-212 and miRNA-132 were markedly upregulated by cAMP. We therefore aimed to identify proteins in the signal transduction pathway upstream of the promoter. Since Creb has been shown to bind to the known CRE-sites of the miRNA-212/132 promoter [184, 185] we were interested in co-activators of Creb, and then particularly in the Crtcs [60].

We identified all three isoforms of Crtcs in the rat insulinoma cell line INS1 832/13 cells and showed that the Crtc1 isoform regulates miRNA-212 and miRNA-132 expression in these cells. Furthermore, using silencing of Crtc1 we could show that Crtc1, is an important regulator of insulin secretion in INS1 832/13 cells.

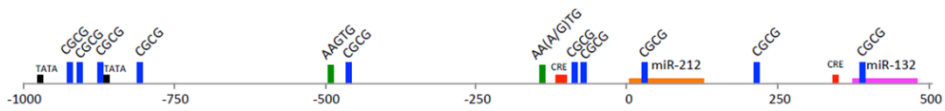

Figure 5
Location of two known CRE motifs (Vo et al. 2005) and putative CGCG and NKE motifs conserved in human, mouse and rat on the promoter of the miRNA212/132 cluster (from Mollet et al 2016).

Dephosphorylation and nuclear translocation of Crtc1 is cAMP-dependent in INS1 832/13 cells.

Crtc1 needs to be dephosphorylated to be able to enter the nucleus and co-activate with members of the Creb/Atf-1-family [60, 151]. We therefore investigated whether cAMP-induction would facilitate the Crtc1 entrance to the nucleus in insulin secreting beta cells and pancreatic islets.

We could show a marked dephosphorylation of Crtc1 at serine-151 after short cAMP-elevating incubation and that this cAMP–stimulated dephosphorylation was enough to enable Crtc1 to enter the nucleus in rat pancreatic or INS1832/13 beta cells. Simultaneously we identified a phosphorylation (activation) of Creb/Atf-1 at Ser-133, in these cells [128].

Siks are involved in the regulation of miRNA-212 and miRNA-132 in insulin secreting beta cells

The Siks are suggested protein kinases of the Crtcs including Crtc1 [145]. Siks are inactivated (phosphorylated) by cAMP induced PkA-activity. We therefore investigated if Siks are involved in the Crtc1-mediated regulation of miRNA-212 and miRNA-132 expression in the insulin secreting beta cell.

We observed that Sik2 (serine-358) and Sik3 proteins were phosphorylated by cAMP induction in pancreatic beta cells and that all SIKs were negative regulators of miRNA-212 and miRNA-132 expression in INS1 832/13 cells [186]. Further, inhibition of all Siks resulted in reduced phosphorylation of Crtc1.

Impact of miRNA-212 and miRNA-132 on Crtc1 expression in INS1 832/13 cells

There is evidence in neuronal tissue of miRNA-212 being implicated in feedback loops with its own regulator [187]. We identified Crtc1 as a putative target of miRNA-132 in rat.

Overexpression of miRNA-212 and miRNA-132 resulted in decreased expression of *Crtc1*, but not *Crtc2* or *Crtc3* mRNAs. After 24h of cAMP induction or 48h

overexpression of miRNA-132 the protein levels of Crtc1 decreased in INS1 832/13 cells.

Expression of miRNA-212 and miRNA-132 and their regulators in human islets

Finally, we investigated expression of miRNA-212, miRNA-132, CRTC1 and SIKs in human pancreatic islets (Human Tissue Lab, 2015). There was a strong correlation between miRNA-212 and miRNA-132 expression, as expected. Moreover, there was a positive correlation between SIK1 and miRNA-132 expression in human pancreatic islets. However, no correlation was observed between CRTC1 and miRNA-212 or miRNA-132 expression.

Summary

I. MiRNA-212 and miRNA-132 modulate insulin secretion and their expressions are induced by cAMP in pancreatic beta cells.

II. Nuclear translocation of Crtc1 is cAMP dependent. Moreover, Crtc1 regulates insulin secretion and co-regulates expression of miRNA-212 and miRNA-132 in the INS1 832/13 cells.

III. Siks regulate nuclear translocation of Crtc1. Siks are involved in the regulation of miRNA-212 and miRNA-132 in INS1 832/13 cells. The expression of SIK1 correlates positively with miRNA-132 levels in human pancreatic islets.

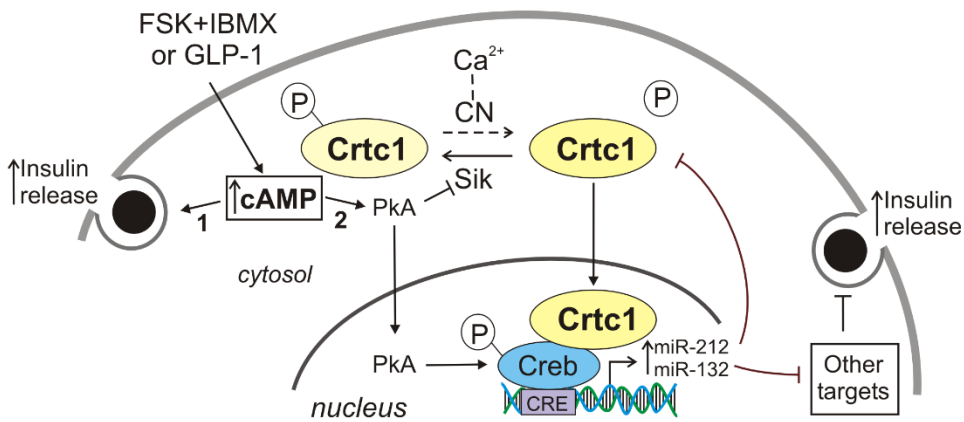

Figure 6
Model figure of SIK and CRTC1-mediated cAMP-dependent regulation of miRNA-212 and miRNA-132 expression and insulin secretion (modified from Malm et al. 2016).

Paper II

Bioinformatic analysis of the rat miRNA-212 and miRNA-132 promoter

Our findings of differentially expressed miRNAs in the diabetic GK-rat made us hypothesize that the differential expression of these miRNAs could be due to a misregulation. [18]. This lead us to perform a bioinformatic analysis of the presumptive promoters of these miRNAs in rat.

We identified putative CM2 or CG-1 motifs, known earlier to interact with transcription/co-transcriptional factor CAMTA, at the presumptive promoter of the miRNA cluster miRNA-212 and miRNA-132 [156, 188-190] (Figure 6.). Furthermore, two putative NKE elements, known as binding sites for NK2-related factors were identified.

CAMTAs and NKX2.2 are regulated by glucose in rat insulinoma cells and human islets, and their gene expression is decreased in diabetic rat islets

CAMTA has never been mechanistically studied before in the context of insulin secretion. Therefore, we aimed to investigate this putative novel transcription/co-transcriptional factor in beta cells. We initially aimed to elucidate the glucose-responsiveness as well as expression of this transcription factor in diabetic islets.

We found both isoforms of CAMTA (CAMTA1 and CAMTA2) as well as NKX2.2, a NK2 homeobox transcription factor [162, 163], to be downregulated in rat pancreatic islets and upregulated in human islets in high glucose. Moreover, the expression of Camta1, Camta2 and Nkx2.2 was downregulated in the diabetic GK rat compared to healthy Wistar rats during glucotoxic conditions (16.7 mM glucose for 24h). This expression pattern persisted for Camta1 and Camta 2, but not for Nkx2.2, after resetting islets to physiological glucose levels.

Expression of the miRNA-212 and miRNA-132 cluster is decreased in GK rat islets under glucotoxicity

We have previously shown mature miRNA-212 and miRNA-132 to be regulated by glucose during short [18, 191] and long [18] incubation in low and high glucose.

We could confirm that miRNA-212 and miRNA-132 are regulated by glucose. In addition, we observed the expression of the pri-miRNA-212/132 transcript to be strongly increased in Wistar rat islets at high glucose. However the elevated expression was significantly less in the GK rat islets.

Expression of miRNA-212 and miRNA-132 and the activity of the miRNA 212/132 promoter are decreased after Camta1 knockdown

We next aimed to explore whether CAMTA regulates the expression of miRNA-212 and miRNA-132 in pancreatic beta cells, since we identified putative motifs known to interact with the CAMTA- [156] and NK2-element transcription factors [164].

We found expression of miRNA-212 to be significantly downregulated, and miRNA-132 to be borderline significantly downregulated after an almost complete silencing of *Camta1* in INS1 832/13 cells. However, no effect on these miRNAs was observed after silencing of *Camta2* or *Nkx2.2*. Further, we found decreased activity from the rat promoter of miRNA-212 and miRNA-132 under cAMP stimulation after silencing *Camta1*.

Camta1 regulates insulin secretion and insulin content in pancreatic beta cells

Our findings that Camta1 is regulated by glucose and differentially expressed in the GK rat islets prompted us to investigate the impact of Camta1 on pancreatic beta cell function.

We observed that silencing of *Camta1* significantly reduced glucose stimulated insulin secretion in INS1 832/13 cells and in rat islets, while the insulin content was increased 3-4 fold under both basal and high glucose concentrations (INS1 832/13 cells).

Involvement of Camta1 in beta cell stimulus secretion coupling

To follow up our observation that Camta1 is involved in the regulation of both insulin secretion and insulin content, we next investigated involvement of this transcription factor in the stimulus-secretion coupling. Camta1 is known to bind calmodulin with high affinity in the presence of Ca^{2+} [154], and we investigated if it is involved in regulation of Ca^{2+} influx and/or exocytosis [54].

We found that Camta1 reduced K^+ -stimulated insulin secretion. This result suggests that the effect of Camta1 is downstream of depolarization. Indeed, voltage-dependent Ca^{2+}-currents, measured using the whole-cell configuration of the patch-clamp technique, was significantly reduced after *Camta1* silencing in INS1 832/13 cells. However, depolarization induced exocytosis, measured as an increase in membrane capacitance, was not affected by silencing of *Camta1*.

Camta1 and Nkx2.2 interactions in insulin secreting beta cells.

As Camtas have been shown to interact with Nk2 transcription factors [157] and we found Nkx2.2 to be downregulated in the diabetic GK rat at 16.7 mM glucose, we aimed to investigate the interactions between Camta1 and Nkx2.2 at low (2.8

mM glucose), intermediate (5.0 mM glucose) and high (16.7 mM glucose) glucose concentrations in insulin secreting cells.

We located Camta1 in both the nucleus and the cytoplasm of INS1 832/13 cells and found the highest number of interactions between Camta1 and Nkx2.2 at 5 mM glucose. Furthermore, we observed less interactions of Camta1 and Nkx2.2 in the nucleus compared to the cytoplasm at low and high concentration of glucose, whereas the number of Camta1-Nkx2.2 interactions did not differ between nucleus and cytoplasm at the intermediate concentration.

Expressions of critical beta cell genes are decreased after Camta1 knockdown in INS1 832/13 cells

To further verify a role of Camta1 in beta cell function, and to rule out an involvement or compensatory effect of Camta2, we investigated the impact of Camta1 in INS1 832/13 on a number of targets of Nkx2.2 in islet cell function [162, 163, 192-194].

We interpreted that silencing of *Camta1* in INS1 832/13 cells resulted in downregulation of genes encoding the islet glucose transporter (*Glut2* or *Scl2a2*), the insulin gene transcription factor (*MafA*), the insulin genes *Ins1*, *Ins2* and the transcription factor *Nkx2.2*. No changes in expression of any of the studied genes were observed when silencing *Camta2*.

Summary

I. Putative CAMTA and NK2-responsive elements are found on the promoter of miRNA-212/132 cluster. The activity of the cAMP-stimulated miRNA-212/132 promoter and the expression of miRNA-212 and miRNA-132 are decreased after *Camta1* knockdown in pancreatic beta cells.

II. Expression of *CAMTA1* is glucose-regulated in insulin-secreting beta cells, and downregulated in diabetic GK rat islets.

III. Knockdown of *Camta1* decreases the voltage dependent Ca^{2+}- currents, reduces insulin secretion and increases insulin content in pancreatic beta cells.

IV. The interaction of Camta1 and Nkx2.2 is glucose-regulated and Camta1 may have an impact on Nkx2.2 regulated expression of beta cells genes, such as *Scl2a2*, *Ins1*, *Ins2, MafA*, as well as *Nkx2.2* itself.

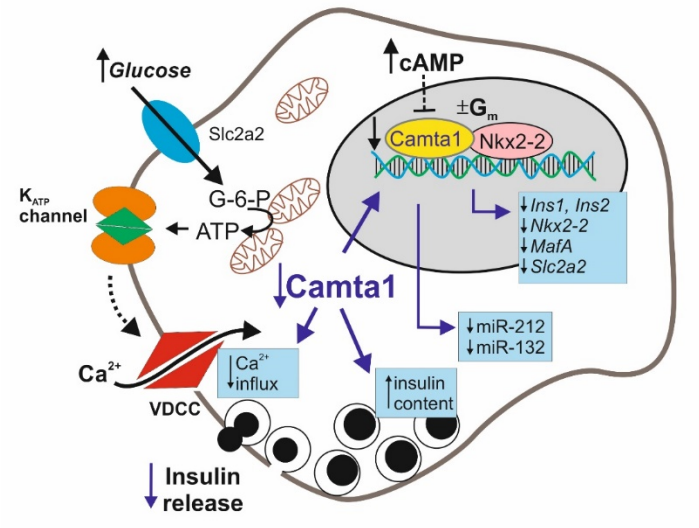

Figure 7
Summary figure paper II. Model figure of the impact of novel transcription/co-transcriptional factor Camta1 on beta cell function (from Mollet et al. 2016).

Paper III

Mapt is a validated target of miRNA-132 in rat pancreatic beta cells

MiRNA-212 and miRNA-132 regulate insulin secretion in pancreatic beta cells [191, 195], but the mechanisms are not clear. Therefore, the aim of this study was to verify targets of miRNA-212 and miRNA-132 in pancreatic beta cells and to mechanistically investigate the involvement of one of these targets in insulin secretion.

In a bioinformatic analysis, we found 249 predicted targets (targetscan.org) [5] of miRNA-212 and miRNA-132 to be conserved in human, mouse and rat, and of these, 21 target genes were differentially expressed in RNA seq data from islets from T2D versus ND human donors. Microtubuli associated protein tau (MAPT), a protein involved in cytoskeletal regulation, and two transcription factors: SRY-box 6 (SOX6) and Insulin enhancer protein 1/Islet LIM homeobox 1 (ISL1) were found to be of special interest [110, 116, 196-198]. The expressions of all three targets were reduced after miRNA-132 overexpression and specifically we verified Mapt as a direct target of miRNA-132 in insulin secreting INS1 832/13 cells.

MiRNA-132 regulates the microtubule network, insulin secretion and insulin content in INS1 832/13 cells

MAPT is known to stabilize and regulate microtubule organization and for its crosstalk with actin [110, 198]. We therefore wanted to investigate the impact of miRNA-132 on the microtubule organization.

Overexpression of miRNA-132 resulted in depolymerization and destabilization of the microtubule network as well as in decreased contact between the microtubule network and the plasma membrane.

Earlier data have shown that overexpression of miRNA-212 and miRNA-132 increase insulin secretion from beta cells [191, 199]. To further elucidate miRNA-212 and miRNA-132 regulation of insulin secretion by these miRNAs we therefore instead downregulated miRNA-212 and miRNA-132 in INS1 832/13 cells, using LNA-212 and LNA-132, and monitored the glucose stimulated insulin secretion. There was no significant effect on the insulin secretion. However, the insulin content was reduced. Moreover, when insulin secretion was related to insulin content, the insulin secretion was increased suggesting an amplification of the secretion process.

Mapt regulates glucose stimulated insulin secretion in earlier stages of the insulin secretion process in INS-1 832/13

Knockdown of Mapt significantly decreased glucose stimulated insulin secretion, without reducing insulin content (data not shown). Depolarization induced insulin secretion using high K^+ was not different in Mapt silenced cells as compared to scramble control. Hence, suggesting that Mapt is involved in stages of the exocytotic process prior to membrane depolarization such as granular mobilization.

Summary

I. Mapt is a direct target of miRNA-132 in rat insulin secreting beta cells and Sox6 and Isl1 are putative targets.

II. Overexpression of miRNA-132 changes the microtubule network appearance, and knock-down of miRNA-132 expression reduces insulin content, but amplifies the secretion process in INS1 832/13 cells.

III. Knockdown of Mapt reduces glucose-stimulated but not depolarization induced insulin secretion.

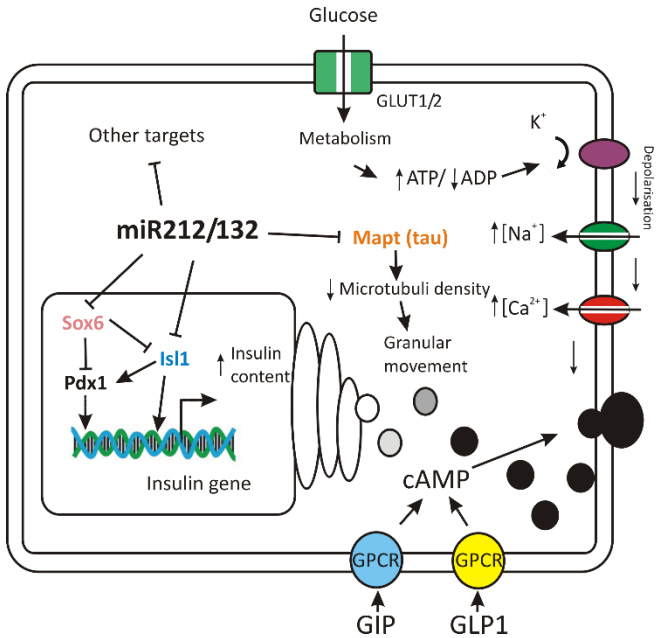

Figure 8
Summary figure paper III. Model figure over the impact of miRNA-212/132 and their targets on insulin secretion from insulin secreting beta cells. (courtesty of H.Malm).

Discussion

Proper regulation of insulin secretion in pancreatic beta cells is crucial for maintaining a balanced blood glucose level [22]. Thus finding new mechanisms and pathways involved in the regulation of insulin secretion from the pancreatic beta cells is of great importance.

We have previously reported miRNAs miRNA-212 and miRNA-132 to be upregulated and specifically regulated by glucose in the pancreatic islets of the non-obese T2D GK-rat [18]. Since we hypothesized that the upregulation of these miRNAs might be caused by a misregulation of the presumptive promoter of this miRNA-212/132 cluster, we have in paper I and paper II of my thesis investigated two transcriptional/co-transcriptional factors presumed to operate in different pathways and found them both to be involved in the regulation of miRNA-212 and miRNA-132 expression and to be essential for beta cell function.

In paper I we investigated cAMP-induced activation of miRNA-212 and miRNA-132 and the regulative effect of these miRNAs on insulin secretion from pancreatic beta cells. We suggest the co-transcriptional factor Crtc1 to partly regulate miRNA-212 and miRNA-132 expression in pancreatic beta cells possibly together with a member of the Creb/Atf-1-family. Furthermore our data together with others [195], show that miRNA-212 and miRNA-132 are involved in the regulation of insulin secretion in insulin secreting beta cells. A function that is investigated in more detail in paper III, where we show that one of the targets of miRNA-132 is Mapt, a protein involved in microtubule organization.

CRTCs are known sensors of hormonal and metabolic signals [200] and CRTC2 has been reported to be involved in beta cell function and proliferation [152]. Here we show Crtc1, however not Crtc2, to be involved in regulating miRNA-212 and miRNA-132. While CRTC1 has been implicated in neuronal and endocrine activities such as long term memory, circadian rhythm, neuroprotection and energy balance in the brain [144, 200-203] this is the first time CRTC1 is reported in beta cell function in the pancreatic beta cell. Interestingly, Crtc1, in addition to having a role in regulation of miRNA expression, is involved in the regulation of insulin secretion through other mechanisms.

Under basal conditions the CRTC protein is sequestered in the cytoplasm in a phosphorylated state by the 14-3-3 proteins. We report all three isoforms of the

AMP-related kinase Sik to phosphorylate Crtc1 at Ser-151 and when silencing Siks we found reduced miRNA-212 and miRNA-132 expression. These findings suggest that Siks regulate miRNA-212 and miRNA-132 expression possibly through phosphorylation of Crtc1 in pancreatic beta cells. Sik2 is claimed to phosphorylate Crtc2 in murine cells [143]. It is likely though that the energy sensing AMP activated protein kinase or any of the other 13 AMPK-related kinases could be considered for the phosphorylation of CRTC1 and CRTC2 in beta cells [111]. SIK2 has also been found to be modulated by the Ca^{2+} sensing protein kinases CaMKI and CaMKIV that phosphorylate SIK2 at Thr484 [204]. In this study we report, as previously shown in adipocytes, phosphorylation at Ser-358 of Sik2 under cAMP or GLP-1 induction in both pancreatic islets and INS1 832/13 cells [205]. Moreover, we show nuclear translocation of Crtc1 under these conditions.

In paper II we identify the Ca^{2+}/calmodulin activated transcription factor (CAMTA) in human and rat pancreatic islets and implicate CAMTA1 as a new player in beta cell function. We find both isoforms of Camta: Camta1 and Camta2 to be chronically downregulated in non-obese diabetic rats compared to non-diabetic Wistar rats and we also show these transcription factors to be regulated by glucose.

Further, we show Camta1 to stimulate insulin secretion. Silencing of *Camta1* reduces both glucose and depolarization induced insulin secretion, suggesting an effect of CAMTA1 downstream of the K_{ATP}-channel. Indeed, silencing of *Camta1* decreased the whole-cell voltage-dependent Ca^{2+} current. Reduced increase in Ca^{2+} is closely linked to reduced exocytosis [85]. Curiously we see no clear evidence of reduced exocytosis (no change in whole cell capacitance) when patching *Camta1* silenced beta cells. The decreased insulin secretion and increased insulin content in CAMTA1 silenced cells, together with that capacitance measurements cannot distinguish between full granule fusion and “kiss-and-run” (or transient granule fusion), suggest increased “kiss-and-run” exocytosis without release of insulin [206].

Evidence from studying Camta1 in long-term memory in the brain claim this transcription factor/co-transcriptional factor to rather regulate transcription of factors associated with electrical excitability (Na^+ and K^+ ion channels) than the exocytotic machinery [207]. However, this cannot be the case in the beta cell since depolarization-induced secretion is likewise affected as glucose stimulated by silencing of Camta1.

Both CRTC1 and CAMTA1 have been shown to be involved in long term memory in brain [201, 207]. Here we show these transcription factors to be implicated in beta cell function. Does CRTC1 and CAMTA1 act together to regulate miRNA-212 and miRNA-132 expression in the pancreatic beta cells? To be able to answer

this question we first need to discuss each of these two transcription factors in a wider context.

We have shown that cAMP facilitates the nuclear translocation of Crtc1 in pancreatic beta cells. But also elevated levels of Ca^{2+} triggers calcineurin (CN)-mediated dephosphorylation of CRTC1 [60, 143]. The cAMP-regulated histone acetylasetransferase CBP, another co-transcriptional activator of CREB, binds to phoshorylated CREB and this interaction has been shown to increase CRTC1 binding to CREB/ATF-1 on the promoter, enhancing transcriptional activity [208]. CaMKIV has also been shown to be a stimulator of CREB activity [131, 138, 209, 210]. However, CREB activity or rather CREB-CBP interaction can be decreased by the Ca^{2+} responsive protein kinase CaMKII that mediates phosphorylation of CREB at Ser-142, thus inhibiting CBP binding [211]. It has been suggested that there is an equilibrium of dephosphorylated/phosphorylated CREB and that this is controlled by ERK/MAPK, CaMK and CN, with interactions between CREB, CBP and CRTC1 [212]. Our findings of Crtc1-mediated Creb activation agrees with this scenario suggestive of a CREB, CBP and CRTC1 equilibrium taking place at the promoter of miRNA-212 and miRNA-132 in the pancreatic beta cells.

What regulates CAMTA expression in the pancreatic beta cell? We show that silencing of *Camta1* reduces Ca^{2+} -influx and Camta1 may interact with Ca^{2+} via its Ca^{2+} /calmodulin domain. However which neuroendocrinal hormone stimulates CAMTA1? CAMTA2 transcription activity is repressed by class II histone deacetylases (HDACs) that promote the binding of histones to the DNA [157]. CaMKII facilitate transcription activity by phosphorylating class II histone deacetylases (e.g. HDAC4), keeping them in the cytosol [213]. It would therefore be likely that the Ca^{2+}-calmodulin dependent protein kinases, the CaMKIIs, are positive regulators of the transcription activity of the calcium/calmodulin activated transcription factor CAMTA1 in the pancreatic beta cells, but this needs to be further investigated. In cardiac muscle Camta2 is shown to translocate between nucleus and cytoplasm as well as associate with the transcription factor Nkx2.5 in the nucleus [157]. We identified the reported diabetes associated transcription factor Nkx2.2 [162, 163] as glucose regulated and differentially expressed in the GK-rat islets. Interestingly we found Camta1 and Nkx2.2 to closely interact with each other at abnormal glucose levels.

It may seem that the expression of miRNA-212 and miRNA-132 promoter, besides the clearly documented cAMP augmentation by us and others [191, 195, 214] is controlled by multiple Ca^{2+} signaling pathways through Crtc1 and/or Camta1. However we have not yet been able to confirm the binding of CAMTA1 to the presumptive CAMTA-motifs at the promoter of the microRNA-212 and 132 cluster. We did register a decreased promoter activity at the miRNA-212/132 promoter when silencing *Camta1*, but only during cAMP induction. Even if we

did see a significant change in miRNA-212 and a trend for miRNA-132, we observed no significant change in the pri-miRNA-212/132 expression when silencing *Camta1* during cAMP induction.

These evidences points towards an indirect regulation by CAMTA1, perhaps through the changes in Ca^{2+}-influx, influencing the dephosphorylation and nuclear entrance of the Ca^{2+} and cAMP-sensitive CRTC1, and subsequently miRNA-212 and miRNA-132 expression in the pancreatic beta cells [143].

In paper 1 we furthermore report Crtc1 as well as miRNA-212 and miRNA-132 to be regulators of insulin secretion in insulin secreting beta cells. While there is a marked impact of Crtc1 on the secretion, probably due to that this co-transcription factor regulate many different genes in the beta cell [200], the increase of insulin secretion after overexpressing miRNA-212 and miRNA-132 is rather minor. The reason for this might be the rapid fine-tuning function, a true characteristics of miRNAs [9]. In order for miRNA-212 and miRNA-132 to be able to execute these fine-tuning mechanisms the system needs to be provided with a feedback mechanism. We believe we may have identified such a mechanism since we see less expression of *Crtc1* and CRTC1 after overexpressing miRNA-132 or after prolonged cAMP induction. Such feedback loops have previously been identified in circadian rhythm as well as cocaine addiction in brain [187, 203].

It is known that miRNA-212 and miRNA-132 essentially target transcription factors [5] and several target proteins have been verified in control mechanisms in other tissues [215]. However, the molecular mechanism by which these miRNAs regulate insulin secretion in the beta cell is not clear. Therefore we aimed to verify novel targets of miRNA-212 and miRNA-132 in pancreatic beta cells and mechanistically investigate the involvement of these targets in insulin secretion.

In paper III we identified MAPT (tau) protein as a conserved target differentially expressed in islets from T2D donors. We report Mapt to be a direct target of miRNA-132 in rat pancreatic beta cells and this protein has recently also been shown to be a target of miRNA-132 in neuronal tissue [216]. The microtubuli-associated protein tau, MAPT, is a stabilizer of microtubule, e.g. it supports smaller microtubule branches and microtentacles [198, 217] . We find this target particularly interesting since MAPT forms neurofibrillary tangles after being aberrant hyperphosphorylated and this protein has been implicated in several neurodegenerative diseases, for example Alzheimer´s [218] [219]. Early findings in pancreatic islets of amyloid fibrill (plaque) formation from the islet amyloid polypeptide (IAPP) show disturbed beta cells and results in decreased beta cell mass [220, 221] . Thus, tau-fibril formation may also disturb beta cell function. Recently, correlation between incidence of neurodegenerative markers and T2D was described [222], suggesting that T2D drives the neurodegeneration in brain independently of Alzheimer´s disease. Several recent reports of increased risk of

T2D incidence in Alzheimer´s patients as well as the tauopathies implicate MAPT (tau) as a player in the pathophysiology of T2D.

When silencing Mapt we experienced a reduced glucose stimulated insulin secretion, but not reduced K^+-stimulated insulin secretion. Thus, suggesting that MAPT has a role in the insulin granular movement. Destabilizing the microtubule network would result in reduced glucose stimulated insulin secretion, which agrees well with that a stabilized microtubule network facilitate the movement of insulin granulaes by motorproteins [100, 101].

When overexpressing miRNA-132 in INS1 832/13 cells, we experience a less dense network of alfatubulin and microtubule branches that are less defined, thicker and seam to loose contact with the plasma membrane. MAPT co-organizes microtubule and actin through binding to both [110]. Although actin does co-exist with the microtubule in the cytoplasm of the cell this cytoskeleton protein polymer mainly delines the plasma membrane and act as a barrier. An overall reduced presence of alfatubulin, markedly at the plasma membrane, when overexpressing miRNA-132 suggests a depolymerization of this protein and lost contact with the plasma membrane and perhaps actin. In fact MAPT has been reported to take part in polymerization and regulation of both microtubule and actin [110].

Further we found evidence of an amplified insulin secretion process after knockdown of miRNA-212 and miRNA-132. This could be explained with increased levels of MAPT protein that would polymerize and tighten the microtubule network and fits well with the microtubule involvement of granular movement explained by Varadi and coworkers [100, 101]. We also noticed that miRNA-212 and miRNA-132, but not Mapt, had an effect on insulin content. We believe this effect is due to other target genes of miRNA-212 and miRNA-132 that regulate insulin biosynthesis, e.g. transcription factors Sox6 and Isl1 [116, 196] that we found downregulated at the mRNA level when overexpressing miRNA-212 and miRNA-132.

To conclude, while Crtc1 is found generally to increase insulin secretion, Camta1 increases the Ca^{2+} of the beta cell and miRNA212/132 regulate insulin secretion through translocation of insulin granule (e.g. Mapt) and insulin biosynthesis (e.g. Sox6 and Isl1).

Concluding remarks

In this thesis I investigated mechanisms involved in transcriptional regulation of the miRNA-212/miRNA-132 cluster and their subsequent regulation of insulin secretion in the pancreatic beta cell through their target proteins. I have identified several novel findings:

I. AMPK-related protein kinases Sik1-3 and co-transcriptional factor Crtc1 regulate miRNA-212 and miRNA-132 expression in pancreatic beta cells, possibly through Creb/Atf-1. Moreover, Crtc1 and microRNA-212/132 regulate insulin secretion in pancreatic beta cells.

II. Camta1 is a novel regulator of insulin secretion in pancreatic beta cells, possibly through regulating Ca^{2+}-influx. Further, Camta1 influences miRNA-212 and miRNA-132 expression and interact with the T2D implicated transcriptional factor Nkx2.2 at non-physiological glucose levels.

III. Mapt is a conserved direct target of miRNA-132 in pancreatic beta cells. Further, miRNA-132 regulate the microtubule network and thereby earlier stages of insulin secretion process through Mapt and putative targets Sox6 and Isl1.

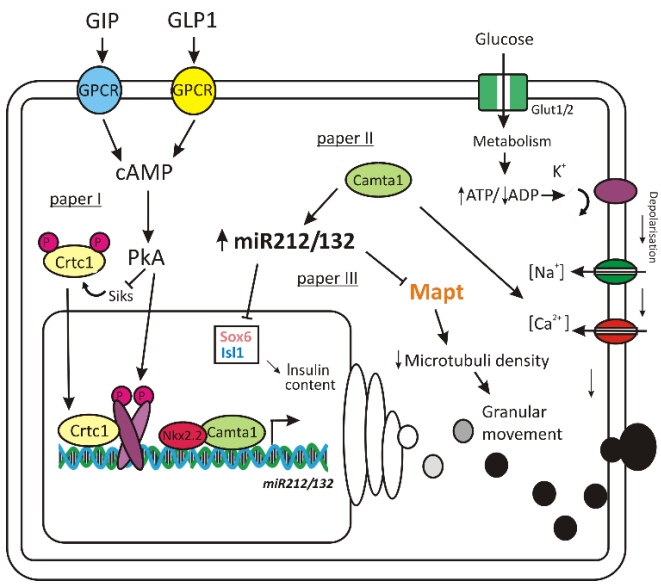

Figure 9
Summary figure paper I-III (courtesty of H.Malm).

Future perspectives

We have reported for the first time Crtc1, Camta1 and miRNA-212 to be regulators of insulin secretion in the insulin secreting beta cells and that miRNA-212 and miRNA-132 are regulated by cAMP and Ca^{2+}, through Crtc1, Camta1 and Creb/Atf-1. Our data points towards an indirect regulation by the *novel* transcription factor in beta cells, CAMTA1, through changes in Ca^{2+}-influx, influencing the dephosphorylation and nuclear entrance of the *novel* Ca^{2+} and cAMP-sensitive coincidence detector of the beta cell, CRTC1 and subsequently miRNA-212 and miRNA-132 expression in the pancreatic beta cells.

So what causes the increased expression of miRNA-212 and miRNA-132 in T2D diabetic GK rats? In paper I we hypothesized that the upregulation of these miRNAs might be caused by a *misregulation* of the presumptive promoter of this miRNA-212/132 cluster and along with our main findings we could show that AMPK-related protein kinases Siks regulate the activity of Crtc1. The upstream regulative molecules of miRNA-212 and miRNA-132 could be screened for T2D associated SNPs. Our sequencing of the promoter of the diabetic GK rat have so far not showed any difference compared to the healthy Wistar rat.

Another explanation could be that the upregulation is a *compensatory* mechanism to control the occurrence of an aberrant phenotype in the diabetic beta cell. Our indications of the existence of a negative feedback loop, where the regulating CRTC1 is reduced in miRNA-132 or miRNA-212 overexpressed samples, indeed points towards such mechanisms. However, to get a general view of the extent of such feed-back/forward mechanisms in the current signaling pathway we need to consider the target proteins of our miRNAs.

We have identified miRNA-132 as a direct strong negative regulator of the microtubule stabilizing protein Mapt (tau) in insulin secreting beta cells. In the pathogenesis of Alzheimer´s disease tau is hyperphosphorylated and forms neurofibrillar tangles (NFT) in dysfunctional neurons [217]. There are as many as 80 phosphorylation sites at the tau protein. GSKIIIβ or tau kinase I (TKI) has together with tau kinase II (TKII) or p35/CDK5 for long been claimed to be the main kinases phosphorylating tau [223]. Also MARK (PAR1 kinases), PKA and CaMKII kinases can phosphorylate tau [224]. As mentioned earlier, Eberhard and colleagues have reported Crtc2 to be involved in beta cell function and proliferation and to be phosphorylated by the energy sensing AMPK-related Sik2

in MIN6-cells [152]. However, recently Sakamaki et al. 2014 reported non-disturbed Crtc2 activity in Sik2 ko mice and instead showed the glucose-responding Sik2, to be the sole kinase, phosphorylating (inhibiting) TKII activity, eventually leading to augmented Ca^{2+} -influx and increased secretion [225]. In short, glucose induced Sik2 would increase the amount of active tau in the beta cell.

I suggest, based on our recent data together with the findings of Sakamaki and colleagues, a model of a cAMP and glucose balanced regulation of insulin secretion, where SIKs are phosphorylated (inhibited) by cAMP and induced by glucose. Downregulation of tau by cAMP induced miRNA212/132 expression and inhibition of tau phosphorylation by glucose induced SIK2, would, in theory, create an equilibrium of the amount of active tau and subsequently microtubule density, affecting granule movement. In the meantime miRNA-212 and miRNA-132 would reduce expression of different transcription factors, e.g. SOX6 and ISL1, affecting insulin biosynthesis. This would together with the positive effect that CAMTA1 and inhibited TKII activity have on Ca^{2+}-influx, and further exocytosis, all together result in increased insulin secretion from the beta cell. An increased expression of miRNA-212 and miRNA-132 would suggest that parts of the balanced insulin regulation system described above does not function properly and hence the hypothesis of the upregulated expression of miRNA-212/miRNA-132 as a *compensatory* mechanism in the type 2 diabetic GK rat.

Our findings of reduced expression of Crtc1 when overexpressing miRNA-132 may be caused by downstream targets of miRNA-212/132. Lately, methylation pattern of the CRTC1 promoter was reported to correlate negatively with phosphorylated tau (p-tau) in Alzheimer´s disease [226] which implicates the miRNA-212 and miRNA-132 co-transcriptional factor CRTC1 in tau- and subsequently microtubule-regulation as suggested by my model above.

MiRNA-212 and miRNA-132 fine-tune multiple targets in a compensatory manner to meet the body´s need of insulin. We should take into account that perhaps at least 5-10 direct target genes of miRNA-212 and miRNA-132 are involved in insulin secretion regulation in the pancreatic beta cell [215]. Also, other miRNAs may be implicated in the above described pathways. Which mechanisms regulate the expression of these miRNAs?

Future work should help us elucidate the involvement of novel targets as well as other miRNAs in insulin secretion in the beta cell. Moreover, our novel findings raise more questions to be answered. Crtc1 is involved in the regulation of insulin secretion from the beta cell through other mechanisms, than miRNA-212/132. Which are these mechanisms? Camta1 regulates Ca^{2+}-influx in the beta cell. In our study we see no effect of GLP-1 on CAMTA1. Which other hormone regulate Camta1? Future work should investigate upstream players in this pathway of

activation/regulation of insulin secretion. CRTC1 and CAMTA1 are expressed in alpha cells and in expression data from human islet donors MAPT expression correlates well with glucagon expression (data not shown). Are we in fact studying regulation of glucagon secretion in the alpha cells? We have elucidated the *activation* of Creb-regulated genes through co-activator Crtc1. However, how is this co-transcriptional activation suppressed? Which molecules may be involved? How would our findings of a negative feedback mechanism between miRNA212/ 132 and Crtc1 be implicated in this suppression?

Further, in humans MAPT (tau) have twelve different transcripts [227]. A common alternative splicing changes the interaction between MAPT (tau) and the cytoskeleton. A target of miRNA-132 in brain, PTBP2, promotes MAPT exon 10 inclusion and thereby a stronger bound to the cytoskeleton. This has been implicated in tauopathies in brain. What is the prevalence of exon 10 inclusion patients with T2DM compared to healthy individuals [228]?

In this thesis I have shown that miRNA-212 and miRNA-132 are likely to have an important function in the insulin secreting beta cells. Moreover, I have identified several, for the beta cell, novel regulating molecules. Future work will determine how these findings help us to better understand how changes in the beta cell lead to the pathogenesis of T2D and hopefully the identified regulating molecules may contribute to the development of new therapeutic drugs.

Populärvetenskaplig sammanfattning

Om man upplever att man oftare blir törstig, att mängden urin ökar mycket och man känner sig tröttare kan det hända att man har drabbats av diabetes. Globalt sett kan så många som 422 miljoner vuxna ha diabetes (WHO, 2016) och av dem är 365 000 svenskar (Hjärt-Lungfonden). Sjukdomen förväntas öka och år 2030 uppskattas 552 miljoner människor i världen vara drabbade av diabetes. De allra flesta, 90 procent har typ 2 diabetes.

Diabetes typ 2 kännetecknas av ökad blodsockerhalt p.g.a. minskad insöndring av det blodsockerreglerande hormonet insulin i blodet och ökad insulinresistens. En riskfaktor är övervikt, men det behöver inte vara så. Det har visat sig att vissa personer har en medfödd (genetiskt) benägenhet att utveckla typ 2 diabetes. Om en person utvecklar typ 2 diabetes beror både på genetik och miljöfaktorer. Ändrad kosthållning och ökad motion kan i vissa fall hjälpa, men ofta behövs farmakologisk behandling. Exempelvis ökar läkemedel med GLP-1 analoger och DPP-4-hämmare insöndringen av insulin och metformin ökar insulinkänsligheten.

Min avhandling rör regleringen av insulininsöndringen till blodet. I vår forskargrupp studerar vi framförallt det maskineri som släpper ut insulin från de insulinproducerande betacellerna i bukspottskörteln, men också hur själva mängden insulin som produceras kan regleras. Vi studerar mikroRNA, som är cellens små finjusterare. MikroRNA är en del av den stora mängden DNA (arvsmassa) i våra celler som aldrig omvandlas till protein. Under senare år har det visat sig att mikroRNA utgör en viktig del av regleringen av genuttrycket, d.v.s. hur stor *mängd* protein som ska bildas av en viss gen, i våra celler. MikroRNA består av korta (20-22 nukleotider långa) RNA-bitar som minskar uttrycket av specifika målproteiner i cellen genom att binda till dess budbärarmolekyler (mRNA) och blockera dem från att bli protein. Det har visat sig att uttrycket, mängden, av olika mikroRNA kan vara förändrat vid sjukdom.

Vår forskargrupp har tidigare sett en förändring i mikoRNA uttryck i den del av bukspottskörteln som tillverkar bl.a. insulin hos råttor med typ 2 diabetes. Jag har studerat två av dessa mikroRNA, miRNA-212 och miRNA-132, för att ta reda på varför de uttrycks mer hos den diabetiska råttan samt hur dessa mikroRNA påverkar de insulinproducerande betacellerna i bukspottskörteln. I de två första delprojekten har vi studerat själva regleringen av uttrycket av miRNA-212 och miRNA-132. Vi kan konstatera att uttrycket av dessa mikroRNA är styrda av bl.a.

hormonet GLP-1, vilket visar att mikroRNA-212 och mikoRNA-132 är viktiga för insulintillverkningen och regleringen av insulininsöndringen till blodet. Dessutom kan vi presentera två nya reglermolekyler i betacellen, CRTC1 och CAMTA1. Vi har upptäckt att CRTC1, som tidigare visats vara inblandad i regleringen av bl.a. dygnsrytmen och långtidsminne, är av stor betydelse för insulinfrisättningen från insulinproducerande celler. Om jag slår ut det här proteinet i de insulinfrisättande cellerna så minskar utsläppet av insulin med ca 50 procent. I samarbete med en forskargrupp i Lund, har vi uttrönt vilka protein i betacellen som möjliggör transport av CRTC1 till och från cellens kärna och därmed regleringen av gener som påverkar insulinfrisättningen från cellen.

Den andra reglermolekylen är CAMTA1. Det här proteinet har tills helt nyligen mest studerats i växtceller och har sammankopplats med cellernas stressreglering. Exempelvis styrs den hormonreglerade mognaden hos frukter delvis via CAMTA1. Vi har kunnat visa att CAMTA1 är av betydelse för betacellens funktion och avgörande för hur lätt betacellen kan triggas att insöndra insulin i blodet. CAMTA1 har också en viss påverkan på regleringen av mikroRNA-212 och mikroRNA-132 uttrycket.

I ett tredje delprojekt har vi identifierat en handfull s.k. målproteiner för de mikroRNA vi studerar. Dessa målproteiner uttrycks annorlunda i den insulinproducerande vävnaden hos personer med typ 2 diabetes, jämfört med friska personer. Dessa proteiner borde alltså på något vis kunna kopplas till typ 2 diabetes. Ett av de målprotein vi tittar på är tau. Sedan tidigare vet vi man att tau kan sättas i samband med sjukdomen Alzheimers. Den naturliga funktionen för tau-proteiner är att stabilisera transportsystemet i cellen. Hos alzheimerpatienten har tau-proteinerna trasslat ihop sig till en spiral vilket hämmar nervcellens funktion. På detta sätt skulle även betacellens funktion kunna påverkas, d.v.s. att insulinsekretionen försämras, betacellerna dör och transportfunktionen påverkas negativt. Vi har kunnat visa att tau verkligen är ett målprotein för mikroRNA-132 i betacellen och att när vi förändrar uttrycket av mikroRNA-132 påverkas mikrotubuli, en del av transportsystemet i cellen. Dessutom, om vi trycker ner uttrycket av tau minskar insulinsekretionen från betacellen.

I denna avhandling har jag visat att mikroRNA-212 och mikroRNA-132 sannolikt har en viktig reglerande funktion i kroppens insulinproducerande betaceller, samt identifierat flera, för betacellen nya reglerproteiner. Detta arbete kommer att leda till en bättre förståelse för hur förändringar i betacellen kan bidra till att typ 2 diabetes uppkommer och kanske kan de identifierade reglermolekylerna bidra till utvecklandet av nya läkemedel.

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