



# LUND UNIVERSITY

## Exocytosis in Type 2 Diabetes- Functional and genetic studies of hormone secretion

Andersson, Sofia A

2012

[Link to publication](#)

*Citation for published version (APA):*

Andersson, S. A. (2012). *Exocytosis in Type 2 Diabetes- Functional and genetic studies of hormone secretion*. Unit of Islet Cell Exocytosis.

*Total number of authors:*

1

### General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117  
221 00 Lund  
+46 46-222 00 00

**Exocytosis in Type 2 Diabetes**  
**-Functional and genetic studies**  
**of hormone secretion**

**Sofia A Andersson**



**LUND**  
UNIVERSITY

**Academic dissertation**

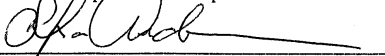
By due permission of the Faculty of Medicine, Lund University, Sweden to be defended at the CRC Auditorium, Entrance 72, Skåne University Hospital, Malmö, on Friday 11<sup>th</sup> of May, 2012, at 13:15 for the degree of Doctor of Philosophy, Faculty of Medicine.

**Faculty opponent:**

Professor Susanne Ullrich  
Department of Internal Medicine,  
University of Tübingen, Tübingen, Germany

Organization LUND UNIVERSITY  Department of Clinical Sciences, Malmö	Document name DOCTORAL DISSERTATION	
	Date of issue 11/5-2012	
	Sponsoring organization	
Author(s) Sofia A Andersson		
Title and subtitle Exocytosis in Type 2 Diabetes - Functional and genetic studies of hormone secretion		
<p>Abstract</p> <p>Type 2 Diabetes (T2D) is characterized by dysregulated beta-and alpha-cell hormone secretion leading to elevated blood glucose levels. Several proteins are crucial in maintaining functional exocytosis of the hormone-containing granules such as Syntaxin1A (Stx1A), SNAP25, Munc-18 (Stxbp1) and the family of Synaptotagmins (Syts). The aim of this thesis has been to investigate functional and genetic events that participate in the exocytotic process.</p> <p>First we show that SNAP25 is essential for cAMP-dependent rapid exocytosis in insulin- secreting cells, and that the effect may be mediated by binding to cAMP-GEFII (paper I). In mouse alpha-cells, SNAP25 and Stx1A display a glucose-dependent localization where immunoneutralization of SNAP25 and Stx1A strongly reduce the exocytotic response (paper II). In paper III, the mRNA expression levels of 23 genes with known function in exocytosis was compared in human beta-cells from non-diabetic (ND) and T2D donors which revealed that Stx1A, Syt4, Syt7, Syt11 and Syt13 is nominally down-regulated in T2D beta-cells and concomitantly correlate positively with glucose stimulated insulin secretion (GSIS) and negatively with HbA1c levels. Furthermore, silencing of Syt4, Syt7 and Syt13 in INS1-832/13 cells correlate with reduced levels of GSIS. In paper IV exocytosis in human beta-cells was studied to generate a risk score consisting of 4 SNPs in or near TCF7L2, KCNJ11, ADRA2A and KCNQ1 that successfully predict reduced exocytosis, calcium-sensitivity, granular docking and GSIS. Finally, in paper V, knock-down of microRNA-335 in insulin-secreting cells was found to increase Stxbp1 expression which correlate with increased exocytosis.</p> <p>Within the scope of this thesis we establish that exocytosis is an important part of glucose homeostasis and that a broad range of factors can influence the exocytotic outcome. In this context, regulation of exocytosis span from the transcription of DNA, translation, transport and function of exocytotic proteins as well as their intricate interactions with one another. All while maintaining the correct cues in response to increased levels of intracellular calcium either amplified by second messengers or triggered by glucose-stimulated coupling. Taken together, extensive research on the molecular mechanisms participating in hormone exocytosis may well contribute to future treatment of the reduced insulin secretion apparent in T2D.</p>		
Key words: Type 2 Diabetes, Insulin, glucagon, alpha-cells, beta-cells, exocytosis, capacitance, SNARE, SNAP25, Syntaxin1A, Synaptotagmin, Munc-18 (Stxbp1), SNP, expression, miRNA		
Classification system and/or index terms (if any):		
Supplementary bibliographical information:	Language English	
ISSN and key title: 1652-8220	ISBN 978-91-86871-97-0	
Recipient's notes	Number of pages 180	Price
	Security classification	

Distribution by (name and address) Sofia A Andersson, CRC, SUS, Entrance 72, 205 02 Malmö, Sweden  
I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature 

Date 5/4-2012

**Exocytosis in Type 2 Diabetes**  
**-Functional and genetic studies**  
**of hormone secretion**

**Sofia A Andersson**



**LUND**  
UNIVERSITY

© Sofia Andersson, 2012

Faculty of Medicine  
Institute of Clinical Sciences  
Department of Islet Cell Exocytosis

Printed in Sweden by Media-Tryck, Lund 2012-04-05

ISSN 1652-8220  
ISBN 978-91-86871-97-0  
Lund University, Faculty of Medicine Doctoral Dissertation Series 2012:35

You live and learn. At any rate, you live.  
- Douglas Adams

# Table of Content

<b>1 ORIGINAL PAPERS</b>	9
<b>2 ABBREVIATIONS</b>	10
<b>3 INTRODUCTION</b>	11
<b>The concept of Type 2 Diabetes and islet hormone secretion</b>	11
Glucose regulation by pancreatic endocrine hormones	11
Type 2 Diabetes	12
Exocytosis and Type 2 Diabetes	12
<b>Secretion and exocytosis of islet hormones</b>	13
Insulin Synthesis	13
Glucagon Synthesis	13
Stimulus-secretion coupling in beta-cells	14
<i>Triggering pathway in mouse beta-cells</i>	14
<i>Ca<sup>2+</sup>-channels involved in hormone secretion</i>	15
<i>Triggering pathway in human beta-cells</i>	15
Amplifying pathway of glucose stimulated secretion	15
Stimulus-secretion coupling in the alpha-cell	16
Functional pools of granules	16
CyclicAMP-dependent exocytosis	17
<b>The exocytotic machinery</b>	19
SNARE-proteins are needed for exocytosis	19
Additional proteins involved in the exocytotic process	20
<b>Genetic Influences on Exocytosis</b>	22
Single nucleotide polymorphism and Genome Wide Association	22
Changed levels of mRNA expression	22
Regulation by microRNA	23
<b>4 MATERIALS AND METHODS</b>	24
Cell culture	24
Transmission Electron Microscopy (TEM)	24
Confocal Laser Microscopy	26
Patch-Clamp and capacitance measurements	26
Quantitative RT-PCR (qRT-PCR)	28
Statistics	28
<i>Association Studies-Relative Risk</i>	29
<i>Statistical analysis of calcium sensitivity</i>	29
<b>5 AIMS AND THE PRESENT INVESTIGATION</b>	30

<b>6 RESULTS AND DISCUSSION</b>	31
<b>Paper I</b>	31
Both full-length and truncated SNAP25 binds to cAMP-GEFII and Rim2	31
Truncation of SNAP25 reduce cAMP-dependent rapid exocytosis	31
cAMP-enhanced GSIS is not altered in SNAP25 <sup>1-197</sup> -expressing cells	32
Discussion paper I	33
<b>Paper II</b>	34
Glucose-dependent localization of SNAP25 and Stx1A in mouse alpha-cells	34
Antibodies against SNAP25 reduce exocytosis of glucagon-containing granules	34
Alpha-cell exocytosis is reduced in presence of antibody against Stx1A	35
Antibody against Stx1A is not associated with a reduced Ca <sup>2+</sup> -current	35
Increased glucose associate with increased number of docked granules	35
Discussion paper II	36
<b>Paper III</b>	37
Decreased expression of exocytotic genes in human islets from T2D donors	37
Correlation of gene expression involved in exocytosis to GSIS and HbA1c	37
Functional effects on insulin secretion	37
Polymorphisms in the <i>RIMS1</i> gene associate with impaired insulin secretion	38
Discussion paper III	39
<b>Paper IV</b>	40
GSIS, but not exocytosis, is reduced in islets from donors with T2d	40
Unaltered number of granules, docked granules and granule diameter in T2D	40
Genetic variants associate with reduced beta-cell exocytosis and docking	41
Genetic risk score for beta-cell dysfunction	41
Discussion paper IV	42
<b>Paper V</b>	44
Glucose-dependent regulation of rno-miR-335, SNAP25 and Stxbp1	44
Increased expression of Stxbp1 after incubation with anti-rno-miR-335	44
Increased exocytosis in anti-miR-335 treated INS1- 832/13 cells	45
Discussion paper IV	46
<b>7 CONCLUDING REMARKS</b>	47
<b>Perspectives</b>	48
<b>8 POPULÄRVETENSKAPLIG SAMMANFATTNING</b>	49
<b>9 ACKNOWLEDGEMENTS</b>	51
<b>10 REFERENCES</b>	52





# 1 Original Papers

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

- I.** Jenny Vikman, Hjalmar Svensson, Ya-Chi Huang, Youhou Kang, Sofia A Andersson, Herbert Y Gaisano and Lena Eliasson (2009) Truncation of SNAP-25 reduces the stimulatory action of cAMP on rapid exocytosis in insulin-secreting cells *Am J Physiol Endocrinol Metab* **297(2)**:E452-46 © American Physiological Society. Reproduced by permission.
- II.** Sofia A Andersson, Morten G. Pedersen, Jenny Vikman and Lena Eliasson (2011) Glucose-dependent docking and SNARE protein-mediated exocytosis in mouse pancreatic alpha-cell *Pflügers Arch* **462(3)**:443-454 © Springer Verlag. Reproduced by permission.
- III.** Sofia A Andersson, Anders H Olsson, Jonathan L Esguerra, Emilia Heimann, Claes Ladenvall, Anna Edlund, Albert Salehi, Jalal Taneera, Eva Degerman, Leif Groop, Charlotte Ling, Lena Eliasson (2012) Reduced expression of exocytotic genes in Type 2 Diabetic human islets, *Re-submitted*
- IV.** Anders Rosengren, Matthias Braun, Taman Mahdi, Sofia A Andersson, Makoto Shigeto, Enming Zhang, Peter Almgren, Claes Ladenvall, Annika Axelsson, Anna Edlund, Morten Pedersen, Anna Jonsson, Reshma Ramracheya, Yunzhao Tang, Jonathan Walker, Amy Barrett, Paul Johnsson, Valeriya Lyssenko, Mark McCarthy, Leif Groop, Albert Salehi, Anna Gloyn, Erik Renström, Patrik Rorsman, Lena Eliasson (2012) Reduced insulin exocytosis in human pancreatic beta-cells with gene variants linked to type-2 diabetes *Diabetes, in press* © American Diabetes Association. Reproduced by permission.
- V.** Sofia A Andersson, Jonathan LS Esguerra and Lena Eliasson (2012) Inhibition of rno-miR-335 enhance rapid exocytosis in insulin secreting cells through increased expression of Stxbp1, *Manuscript*

## 2 Abbreviations

T2D	-Type 2 Diabetes
RP	-Releasable Pool
RRP	-Readily Releasable Pool
ER	-Endoplasmic Reticulum
GLP-1	-Glucagon-Like Peptide 1
DNA	- Deoxyribonucleic acid
RNA	- Ribonucleic acid
mRNA	- Messenger RNA
nt	-Nucleotide; molecules that make up the structural units of <i>e.g.</i> RNA and DNA
GTP	-GuanosineTriPhosphate; nucleotide attached to a ribose sugar with 3 phosphates
ATP	-AdenoTriPhosphate; nucleotide attached to a ribose sugar with 3 phosphates
ADP	-AdenoDiPhosphate; nucleotide attached to a ribose sugar with 2 phosphates
AMP	-AdenoMonoPhosphate; nucleotide attached to a ribose sugar with 1 phosphate
cAMP	-cyclic AMP; second messenger
PKA	-cAMP-dependent Protein Kinase A; enzyme
K <sub>ATP</sub>	-ATP-dependent potassium channel
f	-Femto; prefix meaning 10 <sup>-15</sup>
p	-Pico; prefix meaning 10 <sup>-12</sup>
n	-Nano; prefix meaning one billionth (10 <sup>-9</sup> )
μ	-Micro; prefix meaning one millionth (10 <sup>-6</sup> )
m	-Milli; prefix meaning one thousandth (10 <sup>-3</sup> )
VAMP2	-Vesicle-Associated Membrane Protein 2
Stx1A	- Syntaxin 1A
Stxbp1	-Syntaxin Binding Protein 1, or Munc-18
Stxbp2	-Syntaxin Binding Protein 2, or Munc-13
Syt	-Family of Synaptotagmins
Rab2	-Ras-like GTPase 2
Rim	-Rab2 Interacting Molecule
SNAP25	- Soluble NSF Attachment Protein of 25 kDa
SNARE	- <u>SNAP</u> Receptor
TEM	- Transmission Electron Microscopy

# 3 Introduction

## The concept of Type 2 Diabetes and Islet Hormone Secretion

The prevalence of Diabetes Mellitus is steadily increasing in the world, from about 150 million affected in 1980 to an estimated 340 million in 2008<sup>1</sup>. Common to all patients with diabetes mellitus is a chronic elevation of blood glucose levels in part due to dysregulation of glucose controlling hormones secreted from the pancreas. Hyperglycemia increase the risk of long-term complications related to the damage of blood vessels imposed by high glucose. Both larger and smaller vessels are affected and thus patients with diabetes has a two-fold increased risk of suffering from cardiovascular disease<sup>2</sup> as well as increased risk of *e.g.* chronic kidney disease and damage to the nervous system.

### Glucose Regulation by Pancreatic Endocrine Hormones

Blood glucose is maintained within a narrow range by the release of two hormones from the pancreas namely, insulin and glucagon. The endocrine part of the human pancreas consists of approximately 1 million Islets of Langerhans spread throughout the organ. Each islet contains about 1000-3000 cells including the glucagon-secreting alpha-cells, insulin-releasing beta-cells, somatostatin-secreting delta-cells, pancreatic polypeptide secreting PP-cells<sup>3</sup> and the ghrelin-secreting epsilon-cells<sup>4</sup> which together constitutes 1-2% of the total pancreatic mass. The distribution of cells differs in human versus rodent islets where mouse and rat islets contain 60-80% beta-cells connected by gap junctions in the core of the islet surrounded by a mantle of 20-25% alpha-cells, less than 10% delta-cells and 1% PP-cells that all work as independent units. Human pancreatic cells are randomly distributed consisting fewer beta-cells (48-59%) that does not display a unified signaling pattern, a larger portion of alpha-cells (33-46%) but with delta- and PP-cells similar to rodents<sup>3</sup>. After food intake, blood glucose rises and the beta-cells respond by releasing insulin that lowers the blood glucose by promoting glucose uptake into the liver, fat and muscle cells. At conditions of low blood glucose such as fasting, insulin release is repressed and instead the alpha-cells secrete glucagon that increases blood glucose levels mainly by stimulating release of glucose from the liver. A comprehensive network of blood vessels surrounding the pancreas enables a constant sensing of the blood glucose levels, and likewise serves as way of distribution of the pancreatic hormones to the target cells<sup>5</sup>.

## **Type 2 Diabetes**

Type 2 Diabetes (T2D) is the most common form of Diabetes Mellitus, accounting for over 90% of all diabetes cases globally<sup>6</sup>. T2D develop as a result of both environmental and genetic factors and is defined by high blood glucose levels due to either a failure of target tissues to respond properly to insulin and/or due to failure of the beta-cells to produce enough insulin to sufficiently lower the blood glucose<sup>6</sup>. The development of T2D is strongly correlated with age, a sedentary lifestyle and obesity<sup>7,8</sup> but vulnerability to develop T2D seems also to be strongly inheritable as the lifetime risk of developing T2D when both parents carry the disease is estimated to about 60%<sup>9</sup>. Resistance to the action of insulin in the target tissues is often predominant in obesity however, in most cases of insulin-resistance the decreased sensitivity to insulin is counteracted by enhanced insulin release from the beta-cells<sup>10,11</sup>. T2D develop when the beta-cells fail to adapt to the increased demands, either due to inherent or acquired defects.

## **Exocytosis and Type 2 Diabetes**

Several studies have shown that impaired insulin secretion occurs already before the onset of T2D<sup>12-14</sup> and that the basal levels of glucagon secretion is increased<sup>15</sup>, which indicate that processes involved in hormone secretion are crucial in the development of the disease. The hormonal release from alpha-and beta-cells occurs in several steps culminating in the exocytotic process. During exocytosis, the hormone-containing granules are transported to the cell surface, dock, prime and subsequently fuse with the plasma membrane thereby rendering the lumen of the granule open to the extracellular environment and thus, the hormones are secreted. Docking occurs prior to priming where the granule attaches to the plasma membrane, a process suggested to be aided by the interaction of the plasma membrane bound syntaxin 1A (Stx1A) to the granular protein granuphilin<sup>16</sup>. Several proteins are known to be involved in the priming process such as the formation of the SNARE complex needed for fusion. The SNARE-complex is assembled by the interaction of Stx1A and SNAP25 at the plasma membrane, with the granular-associated VAMP2<sup>17</sup>. Upon Ca<sup>2+</sup>-influx primed granules are released, which is suggested to be regulated by the activity of Ca<sup>2+</sup>-sensing proteins named synaptotagmins (Syt) that interacts with the SNARE-proteins<sup>18</sup>.

Insulin release is biphasic with a first rapid peak lasting 5-10 minutes followed by a second lower sustained long-term secretion. It is suggested that the rapid peak reflects instant release of insulin granules primed at the plasma membrane<sup>19,20</sup>. Patients with T2D characteristically display reduced or complete absence of the first phase which is suggestive of impaired fusion of primed granules, signifying the importance of functional exocytosis<sup>21</sup>. In addition, it has been shown that proteins related to the exocytotic process are reduced in islets from donors with T2D<sup>22</sup>.

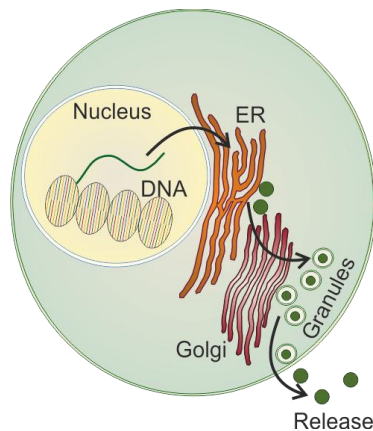
# Secretion and Exocytosis of Islet Hormones

## Insulin synthesis

Insulin is a hormone of 6 kDa which consists of two straight peptide chains linked together by two disulphide bridges. It is originally expressed from the insulin gene (*INS*) in human pancreatic beta-cells<sup>23</sup> as preproinsulin. While in the Endoplasmic Reticulum (ER), the preproinsulin is immediately processed into proinsulin which buds of the ER and is guided towards the Golgi apparatus where the disulfide linkages are established leading to the folded conformation of the proinsulin molecule (Fig 1). Further, while being transported through the Golgi apparatus, the proinsulin is modified by enzymes whereby the final insulin molecule is formed. As the secretory granule mature, insulin becomes associated with zinc which forms the dense central core of the granule. The major part of all proinsulin processed in the Golgi apparatus (99%) results in storage granules for regulated insulin release, where the insulin is retained within the granules as they are transported towards the cell plasma membrane and subsequently released by exocytosis. The remaining 1% of proinsulin escapes storage in granules whereby proinsulin maintains a low rate of constitutive insulin secretion<sup>24</sup>.

## Glucagon synthesis

Glucagon in its final form is a hormone of 3.5 kDa which consist of one single, straight-chain peptide<sup>24</sup>. It is originally expressed from the preproglucagon gene (*GCG*) in human pancreatic alpha-cells<sup>25</sup>. The gene is however expressed also in the intestinal L-cells, but the processing of the preproglucagon differs between the two cell types<sup>26-29</sup> where the alpha-cells cleaves preproglucagon into glucagon<sup>30</sup>, and the L-cells produce glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2) and glicentin<sup>31</sup>. As in the case of all peptide hormones, the glucagon molecule is synthesized in the ER and guided to the Golgi apparatus for packaging into release-competent granules (Fig 1).



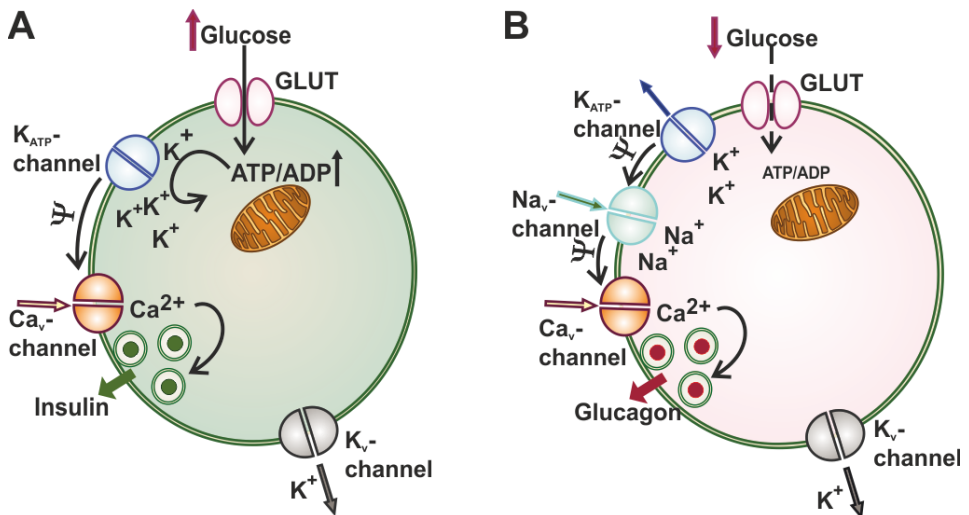
**Fig 1** The secretory pathway of peptide hormonal secretion

## Stimulus-secretion coupling in the beta- cell

The endocrine cells of the Islet of Langerhans are electrically active which is tightly linked to exocytosis of the hormone-containing granules (Fig 2). The cells express glucose transporters (GLUT) through which glucose enter by facilitated diffusion<sup>32</sup>. Upon glucose-stimulation, the beta-cells start generating a characteristic pattern of slow membrane oscillations upon which bursts of  $\text{Ca}^{2+}$ -dependent actions potentials are superimposed<sup>33</sup>. The subsequent increase in intracellular  $\text{Ca}^{2+}$  evokes the  $\text{Ca}^{2+}$ -dependent exocytosis<sup>34</sup>.

### Triggering pathway in mouse beta-cells

The resting potential (-70 mV) of the mouse beta-cell is maintained by an inward rectifier  $\text{K}^+$  -channel ( $\text{K}_{\text{ATP}}$ -channel). The  $\text{K}_{\text{ATP}}$ -channel consists of the sulfonylurea receptor 1 (SUR1) and Kir6.2 subunits: ATPase activity at SUR1 increases  $\text{K}_{\text{ATP}}$ -channel opening whereas binding of ATP to Kir6.2 closes the channel<sup>35</sup>. Glucose entering the beta-cell is metabolized by the mitochondria that utilize glucose to synthesize ATP on the expense of ADP which leads to closure of the  $\text{K}_{\text{ATP}}$ -channels (Fig 2A). The subsequent accumulation of positively charged  $\text{K}^+$ -ions retained within the cell depolarize the cell membrane<sup>36</sup>. When blood glucose concentration exceeds 7 mM the  $\text{K}_{\text{ATP}}$ -induced depolarization following glucose metabolism is sufficient to reach the threshold potential ( $\sim -50$  mV)<sup>37</sup> where downstream voltage-dependent  $\text{Ca}^{2+}$ -channels activate<sup>37,38</sup> and the concomitant influx of  $\text{Ca}^{2+}$  triggers exocytosis of insulin granules<sup>39</sup>. Influx of  $\text{Ca}^{2+}$  is in turn primarily limited by repolarization mediated by efflux of  $\text{K}^+$  through the  $\text{K}_{\text{v}2.1}$  voltage-dependent channels<sup>40</sup>. The glucose uptake by insulin-sensitive tissues in response to insulin restores the extracellular glucose to the normal concentration around 4-5 mM, whereby the glucose uptake into the beta-cell is reduced. Consequently, the  $\text{K}_{\text{ATP}}$ -channels re-open whereby the bursts of action potentials are terminated, the membrane potential return to -70 mV and insulin secretion is inhibited.



**Fig 2** Stimulus-secretion coupling in **A)** beta-cells and **B)** alpha-cells

### *Ca<sup>2+</sup>-channels involved in hormone secretion*

Unstimulated beta-cells maintain a low intracellular Ca<sup>2+</sup>-concentration of 50-100 nM creating a 10 000-fold gradient of Ca<sup>2+</sup> compared to the extracellular level<sup>41</sup>. The major factor of the glucose-induced rise in intracellular Ca<sup>2+</sup> is determined by the opening of voltage-gated Ca<sup>2+</sup>-channels (Ca<sub>v</sub>-channel) in the plasma membrane. The Ca<sub>v</sub>-channels are built up by four subunits namely the alpha<sub>1</sub>, beta, gamma and alpha<sub>2</sub>/delta. The alpha<sub>1</sub> subunit forms the Ca<sup>2+</sup>-conducting pore and contains the voltage sensor, the selectivity filter for Ca<sup>2+</sup> and the activation and inactivation gates. Based on the primary structure of the alpha<sub>1</sub> subunit, the Ca<sub>v</sub>-channels are divided into four families: Ca<sub>v</sub>1-4, where Ca<sub>v</sub>1 and Ca<sub>v</sub>2 are high voltage-activated channels found in human and mouse islets whereas Ca<sub>v</sub>3 is low voltage-activated channels found in human islets and in the diabetic NOD mouse. Depending on the type of current arising from the channel activation, the Ca<sub>v</sub>-channels are further subdivided: Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 are the primary L-type Ca<sup>2+</sup>-channels in mouse, rat and human islets. The function of Ca<sub>v</sub>2.1, also known as P/Q-type channel, is not clear in the mouse islets, but has been shown important in human beta-cell exocytosis. N-type channels (Ca<sub>v</sub>2.2) functions in INS-1 cells, mouse, and human islets, whereas Ca<sub>v</sub>2.3, an R-type channel, is important for insulin secretion in both mouse beta-cells and INS-1 cells. The Ca<sup>2+</sup>-channel subtypes Ca<sub>v</sub>3.1-3.3 are T-type channels involved in INS-1 cells where Ca<sub>v</sub>3.2 is the suggested T-type channel in human islets<sup>42,43</sup>.

### *Triggering pathway in human beta-cells*

The direct triggering pathway in human beta-cells differ from that of rodent beta-cells in that: 1) a Na<sup>+</sup>-channel participates in generating the action potential, 2) the P/Q-type Ca<sup>2+</sup>-channel rather than the L-type initiate action potential, 3) the R-type channel is not expressed, and 4) a BK-channel primarily acts as the repolarization channel. At glucose above ~6 mM, closure of the K<sub>ATP</sub>-channel depolarizes the membrane to potentials above -55 mV. T-type channels activate at potential higher than -60 mV, further leading to activation of L-type Ca<sup>2+</sup> channels and voltage-gated Na<sup>+</sup>-channels. This creates a depolarization sufficient to activate P/Q-type Ca<sup>2+</sup>-channels, which directly trigger exocytosis of insulin granules. The electrical activity within the burst of action potential firing is primarily limited by efflux of K<sup>+</sup> through the large conductance Ca<sup>2+</sup>-activated BK channels closely co-localized to Ca<sup>2+</sup>-channels, whereas K<sub>v</sub>2.1 does not contribute to the same extent as in mouse beta-cells<sup>42,44</sup>.

### **Amplifying pathway of glucose stimulated secretion**

Glucose can further enhance insulin secretion via mechanisms unrelated to the K<sub>ATP</sub> channels, indicating that a pathway other than the triggering pathway can also contribute to exocytosis. Several molecules have been suggested in this metabolic pathway such as glutamate<sup>45</sup>, ATP, GTP, malonyl-CoA<sup>46</sup> and NADPH<sup>47</sup>. However, this amplifying pathway remains silent as long as the intracellular level of Ca<sup>2+</sup> is not raised by the triggering pathway and rather appears to optimize the secretory response<sup>41</sup>.

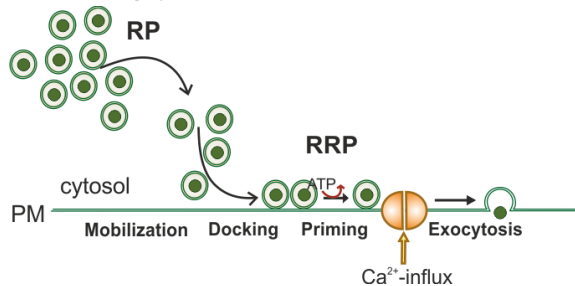


## Stimulus-secretion coupling in the alpha-cell

Escalating glucose concentrations results in a concentration-dependent acceleration of glucose metabolism in both alpha- and beta-cells<sup>48</sup> yet, glucagon secretion is inhibited at glucose concentrations by which insulin secretion is stimulated. This is due to that the direct triggering pathway of glucose in the mouse alpha-cell differs from that in the mouse beta-cell; the alpha-cell contain high voltage-dependent Na<sup>+</sup>-channels activating at -30 mV with a half-maximal inactivation ( $V_{1/2}$ ) of  $\sim$ -50 mV whereas Na<sup>+</sup>-channels of the mouse beta-cell has a  $V_{1/2}$  of  $\sim$ -100 mV downstream from the K<sub>ATP</sub>-channels<sup>49</sup> (Fig 2B). The Ca<sup>2+</sup>-channels activated following glucose-stimulation also differs to those activated in the mouse beta-cell. Increased membrane potential caused by the closure of the high-sensitive K<sub>ATP</sub>-channels following glucose metabolism at blood glucose levels above 7 mM will inactivate the Na<sup>+</sup>-channels in the alpha-cell and inhibit secretion. However, blood glucose lower than 5 mM at which ATP-production ceases (due to lower glucose metabolism) will not close the K<sub>ATP</sub>-channels as efficiently. This creates a membrane potential where partly open K<sub>ATP</sub>-channels maintain a window of potential where opening of low-voltage-activated T-type Ca<sup>2+</sup>-channels (-60 mV) will produce a small depolarization optimal for activation of the Na<sup>+</sup>-channels. Influx of Na<sup>+</sup> will additionally depolarize the plasma membrane at which primarily downstream high-voltage-activated N- and L-type Ca<sup>2+</sup>-channels activate<sup>50,51</sup>. The Ca<sup>2+</sup>-influx evoke exocytosis of glucagon-containing granules<sup>52,53</sup>. It is debated whether glucose or insulin is the main inhibitor of glucagon release<sup>39,54-56</sup>. In one study performed in mouse islets, glucagon secretion peaks at 0 mM glucose, reduces significantly at 4 mM and is totally inhibited at 8 mM glucose. In the same samples, insulin secretion is not significantly detectable below 7 mM glucose<sup>56</sup>. Thus, glucagon secretion is reduced due to a direct effect of glucose rather than an inhibitory effect of insulin.

## Functional pools of granules

The mouse beta-cell contains more than 10 000 insulin granules<sup>57,58</sup> differently positioned within the cell<sup>59</sup> (Fig 3). Granules primed to the plasma membrane are release-competent and fuse instantly upon Ca<sup>2+</sup>-influx and hence are termed the Readily Releasable Pool (RRP). The RRP constitutes about 50-100 granules of the  $\sim$ 600 granules docked at the plasma membrane. Thus, the larger proportion of the granules are retained either in the cytosol or docked at the plasma membrane and it is postulated that this Reserve Pool (RP) is responsible for granular refilling of the RRP<sup>58,59</sup>. A single mouse alpha-cell contains about 7300 glucagon granules, also located in different pools following synthesis, where the RRP constitute about 120 granules<sup>53,60</sup>.



**Fig 3** Flow-chart of the functional pools of granules prior to Ca<sup>2+</sup>-dependent exocytosis.

Refilling of RRP from the RP involves granular transport, docking and priming of granules at the plasma membrane. The transport of granules from the Golgi network towards the plasma membrane is dependent on cytoskeletal components such as the actin filaments and microtubules. Microtubules are present throughout the whole cell, and disruption of the network in beta-cells has been shown to inhibit insulin secretion<sup>61</sup>, a feature also shown when obstructing the microtubule-associated molecular motor kinesin 1<sup>62</sup>. A dense network ~50-300 nm thick consisting of actin filaments is located underneath the plasma membrane, and it seems this network is reorganized in functional exocytosis as it has been demonstrated that interrupting the web stimulates insulin secretion in beta-cells<sup>63</sup>. Granular transport along the actin filaments has been shown to involve motor protein myosin 5A interaction with Rab27a and the synaptotagmin-like protein Slac2c/MYRIP<sup>64</sup>. Docking proceeds priming and is the process whereby the granules attach to the plasma membrane. Priming is a Ca<sup>2+</sup> - dependent process as discussed below. Further, the intragranular environment need to be acidified by pumping of H<sup>+</sup> into the lumen of the granule through a V-type ATPase thus, ATP is needed for the priming process. The granular electrical gradient during the acidification is maintained by Cl<sup>-</sup>-influx through ClC-3 Cl<sup>-</sup>-channels<sup>65</sup>.

The first and second phase of insulin secretion is suggested to reflect instant release of granules from the RRP and refilling of the RRP by granules from the RP, respectively<sup>19,20</sup>. Patients with T2D characteristically display reduced or complete absence of the first phase which is suggestive of impaired fusion of the granules from the RRP with the plasma membrane<sup>21</sup>. In mouse beta-cells, granules of the RRP are tethered close to the L-type Ca<sup>2+</sup>-channels via interaction of the SNARE-complex with the L-loop, separating repeats II-III in the alpha-subunit of the channel<sup>66</sup>. Upon channel activation, these granules thus experience a high local level of Ca<sup>2+</sup> where half-maximal stimulation of exocytosis is achieved at 17 μM Ca<sup>2+</sup><sup>67</sup>. The granular refilling of the RRP from the RP is mainly stimulated by Ca<sup>2+</sup>-influx through the R-type Ca<sup>2+</sup>-channels<sup>68</sup> and proceeds at a slower rate than that of instant release from the RRP<sup>67</sup>. However, influx of extracellular Ca<sup>2+</sup> has also been shown to stimulate release from intracellular stores in the ER<sup>69</sup> and it has been hypothesized that refilling of granules to the RRP may be facilitated also by intracellular Ca<sup>2+</sup> release<sup>70</sup>.

### **CyclicAMP-dependent exocytosis**

In presence of glucose, exocytosis can be further enhanced by second messengers such as the cAMP messenger system. Glucagon, GLP-1 and GIP are all peptide hormones that potentiate insulin secretion via this pathway. cAMP activate the enzyme PKA (cAMP-dependent protein kinase) which in turn, regulate the activity of selected targets by adding a phosphate group transferred from ATP<sup>71</sup>. In beta-cells, the PKA-dependent pathway has been shown to enhance exocytosis by increasing the influx of Ca<sup>2+</sup> through the L-type Ca<sup>2+</sup>-channels<sup>72</sup>, but also by stimulating the refilling of RRP from the RP<sup>73</sup>. In addition, potentiation of the exocytotic process involves PKA-independent stimulation via the low-affinity cAMP-sensor protein cAMP-GEFII, which has been shown to interact with Rim<sup>74</sup> and SNAP25<sup>75</sup> and facilitate granule priming<sup>76</sup>. Inhibition of insulin secretion, on the other hand, can be mediated by paracrine somatostatin signaling via binding to the somatostatin receptor<sup>77</sup> and neuroendocrine signaling by

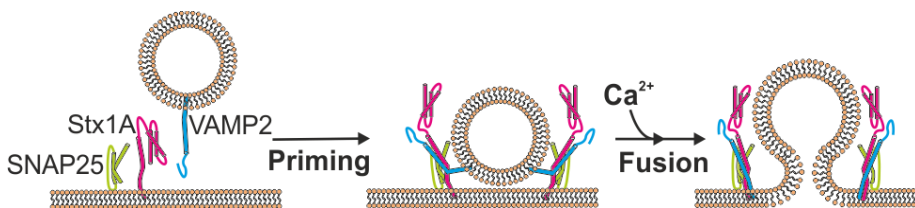
adrenalin binding to the alpha2A-adrenergic receptor(ADRA2A). Signaling via the ADRA2A receptor decreases the levels of cAMP which has been shown to repolarize the plasma membrane in mouse<sup>78</sup> and directly affect exocytosis in rodent and human cells<sup>79</sup>.

In the alpha-cell, cAMP-dependent exocytosis is associated to Ca<sup>2+</sup>- influx through L-type Ca<sup>2+</sup>-channels. In the absence of cAMP, exocytosis is controlled by influx through N-type Ca<sup>2+</sup>-channels<sup>52</sup>. GLP-1 is conversely shown to inhibit glucagon secretion whereas adrenalin potentiates glucagon release via the ADRA2B receptors. One hypothesis for this discrepancy stipulates that the alpha-cell contains few receptors for GLP-1 but a larger content of adrenergic receptors. Hence, signaling via GLP-1 increases the intracellular cAMP levels to a lower extent than signaling via adrenalin. Low levels of cAMP activate the highly sensitive PKAI enzyme, whereas the enlarged cAMP increase induced by adrenaline will also enable activation of the low affinity cAMP sensors PKAII and cAMP-GEFII. PKAI inhibits the N-type Ca<sup>2+</sup>channels thus, GLP-1 inhibits glucagon secretion. PKAII augment granular refilling of the RRP from the RP and cAMP-GEFII enhances priming hence, both pathways initiated by adrenaline signaling stimulate glucagon secretion<sup>80</sup>.

# The exocytotic machinery

## SNARE-proteins are needed for exocytosis

Fusion of granules with the plasma membrane during  $\text{Ca}^{2+}$ -dependent exocytosis requires formation of SNARE-complexes. The SNAREs comprise one protein integrated in the granular membrane, VAMP2, and two proteins associated to the cell-membrane, Stx1A and SNAP25 (Fig 4). Stx1A is integrated at the plasma membrane through a C-terminal trans-membrane domain and contains a cytosolic regulatory domain at the N-terminal. SNAP25 is anchored to the plasma membrane through palmitoylation of four cysteine residues in the middle of the protein. Two alpha-helices of SNAP25 assembles with Stx1A and VAMP2 thereby forming a four-helical bundle that clasp the granule to the membrane. Upon  $\text{Ca}^{2+}$ -entry the SNARE-complex changes conformation thereby creating the force needed for the granule to fuse with the plasma membrane<sup>81</sup>. Electrical activity needed for granular docking and release may also depend on SNARE-interaction<sup>82</sup>. Both SNAP25 and Stx1A has been proposed to modulate the voltage-gated  $\text{Ca}^{2+}$ -channels<sup>21,83</sup> as well as other ion channels<sup>40</sup>.



**Fig 4** The SNARE-complex assembly preceding fusion of granules. Illustration modified from Fig 2 in “Exocytosis in insulin secreting cells -Role of SNARE-proteins” (2008), kindly provided by Jenny Vikman.

In beta-cells, Stx1A and SNAP25 proteins are arranged in clusters along the plasma membrane<sup>84,85</sup> in close association with the insulin granules and the number of clusters are reduced in the diabetic GK-rat<sup>84</sup>. In accordance, the GK-rat display reduced expression of SNAP25 and Stx1A<sup>86</sup>. It has further been shown that following exocytosis in neuroendocrine cells, syntaxin molecules diffuse away from the site of fusion<sup>87</sup> and localize to the ER and Golgi region<sup>88</sup>. The transport of Stx1A to the plasma membrane may require Stxbp1 (Munc-18), and Stx1A might in turn be required for transport of SNAP25<sup>88</sup>. In pancreatic beta-cells, re-localization of SNAP25 from the plasma membrane to the cytosol has been shown to reduce exocytosis and insulin secretion<sup>89</sup>.

The SNARE-proteins are vital for optimized insulin secretion in beta-cells (for inept reviews see<sup>21,82,90</sup>). For instance, a Stx1A null mice display impaired glucose tolerance and decreased insulin secretion caused by reduced docking and priming<sup>91</sup>. In contrast, transgenic mice overexpressing Stx1A are also glucose intolerant and have reduced exocytosis<sup>92</sup>. Furthermore, the blind-drunk (*Bdr*) mouse, which has a point mutation in *Snap25b* that increases the binding-affinity to Stx1A, has reduced granular refilling in the beta-cells<sup>93</sup>.

## Additional proteins involved in the exocytotic process

Apart from the assembly of the SNARE complex, many other proteins are needed for exocytosis of the granules such as Stxbp1, Munc-13, the Rims proteins, and the family of Synaptotagmins (Syt)<sup>21,94, 68, 70-72</sup>.

The Sec1/Munc18 (SM) proteins contribute to the Ca<sup>2+</sup>-sensing of exocytosis<sup>94</sup>. Stxbp1 (or Munc18-1) has been shown to redistribute to the plasma membrane upon glucose stimulation<sup>95</sup> and is an important regulator of exocytosis. The N-terminal of Stx1A must be moved in order to allow access of SNAP25 to the core domain of Stx1A, and Stxbp1 is suggested to bind to the folded conformation of Stx1A during docking<sup>96,97</sup> thereby preventing downstream exocytotic events. Upon Ca<sup>2+</sup> influx Stxbp1 dissociates from Stx1A thereby allowing formation of the SNARE complex and subsequent insulin granule exocytosis<sup>98-100</sup>. However, there are also studies showing that Stxbp1 is capable of binding to the N-terminal of Stx1 in the open configuration<sup>101</sup> thereby promoting fusion<sup>102</sup>. The opposing modes of targeting is believed to be regulated by different phosphorylation/dephosphorylation actions on Stxbp1<sup>103</sup>. Adding further to the diversity; Stxbp1 binding to the assembled complex of Stx1A/SNAP25 has been shown, which promotes fusion in neuronal cells<sup>104</sup>. It appears that Stxbp1 can interact with Stx1A in three general regions: The N-terminus, the three-helix bundle, and the core SNARE domain (H3) of Stx1A<sup>105</sup>. In addition, Stxbp1 is also suggested to be needed in the transport of Stx1A from the ER to the cell membrane<sup>88</sup>.

Rim proteins contribute to the Ca<sup>2+</sup>-triggering of exocytosis<sup>106</sup> and contains an N-terminal domain that interacts with both the active form of Rab3 and Munc13<sup>82</sup>. The Rab family are controlling different steps in the secretory pathway<sup>107</sup> by modulating the assembly of the SNARE complex<sup>108</sup>. The Rim proteins also contain Ca<sup>2+</sup>-binding C<sub>2</sub>-domains that can interact with Snap25, Syt1 and the Ca<sup>2+</sup>-channels<sup>109</sup>. Specifically, one isoform of Rim, Rim1 has been demonstrated to influence the activity of the L-type Ca<sup>2+</sup>-channels<sup>110</sup> and the isoform Rim2alpha is suggested to be crucial for granular docking and priming<sup>111</sup>. Rim proteins are in turn regulated by cAMP-GEFII in a cAMP-dependent, PKA-independent fashion<sup>74</sup>.

The family of Syts consists of at least 16 members with different functions depending on cell type (inept review see, <sup>112</sup>); multiple Syts are often present within the same cell acting independently or in concordance on different steps of granular trafficking<sup>113</sup>. The domain structure consists of a short N-terminal sequence within the granular lumen, a single trans-membrane domain, and a cytoplasmic sequence containing two calcium-binding C<sub>2</sub> domains; C<sub>2</sub>A and C<sub>2</sub>B. Upon binding Ca<sup>2+</sup>, the calcium-binding pocket is altered<sup>114</sup> which enhances interactions with phospholipids and proteins of the SNARE complex<sup>115</sup>. The Syt1-3, 5-7 and 9-10 display Ca<sup>2+</sup>-dependent phospholipid binding to the C<sub>2</sub>A-domain, whereas the other members do not<sup>18,116</sup>. Further, Syt5, 9 and 13 has been detected in primary beta-cells<sup>106,117,118</sup> and Syt4 expression has been shown in clonal beta-cell lines, rat islet cells<sup>119</sup> and in rat alpha-cells<sup>120</sup>. It is meant worthy that Syt1, 4 and 7 are also expressed in *Drosophila* and *C. elegans*, suggesting their association in vital processes<sup>121</sup>

Syt1 is the best characterised member which is primarily located in neuronal cells and binds to both the intact SNARE complex<sup>122</sup> as well as to heterodimers of either the C-terminal of Stx1 or SNAP-25<sup>123,124</sup>. The binding is enhanced by  $\text{Ca}^{2+}$ <sup>125</sup> and successful exocytosis has been shown to depend on the interaction of Syt1 with both  $\text{Ca}^{2+}$  and SNAP25<sup>126</sup>.

Syt4 has been more extensively studied in neuronal cells and contains an aspartate to serine substitution in the C<sub>2</sub>A-domain rendering it less efficient in  $\text{Ca}^{2+}$ -binding<sup>127</sup>. Nevertheless, Syt4 has been proposed as a  $\text{Ca}^{2+}$ -sensor because it binds phospholipids in the presence of  $\text{Ca}^{2+}$ <sup>128</sup>. However, the functions of Syt4 in  $\text{Ca}^{2+}$ -dependent release remains to be established as it has been shown to inhibit<sup>129</sup>, modulate<sup>130</sup> or stimulate exocytosis. In the latter case, the C<sub>2</sub>A-domain was found to bind to Stx1 in a non- $\text{Ca}^{2+}$ -dependent manner and instead  $\text{Ca}^{2+}$  was bound at the C<sub>2</sub>B-domain<sup>131</sup>.

Syt7 is established as a crucial player in  $\text{Ca}^{2+}$ -dependent regulation of insulin secretion and Syt7 knockout mice display impaired glucose tolerance and lowered basal-and glucose-induced insulin levels<sup>132</sup>. In alpha-cells, Syt7 binds to Stx1A<sup>133</sup> and may be the major  $\text{Ca}^{2+}$  sensor in the  $\text{Ca}^{2+}$ -dependent exocytosis<sup>134</sup>.

To my knowledge, Syt11 and Syt13 have not previously been studied in the beta-cell. Syt11 is closely related to Syt4 in that it shares the same amino-acid substitution. Syt13 is amongst the later synaptotagmins discovered, primarily in the brain and differs in that it does not contain an intra-granular N-terminal and the C<sub>2</sub>-domains lack most of the residues involved in  $\text{Ca}^{2+}$ -binding<sup>135</sup>.

## Genetic Influences on Exocytosis

The DNA strand contains approximately three billion base pairs; which were sequenced in the correct order in the HUGO project 2001<sup>136,137</sup>, further organized into 22 autosomal chromosome pairs and two sex-specific chromosomes (X and Y). Within the genome lays approximately 20-25 000 protein-coding genes<sup>138</sup>. The DNA serves as a blueprint; the two strands separate so that the template strand can be used for transcription into messenger RNA (mRNA) which is then transported into the cytosol. The immature protein translated by the ribosome as it travels along the mRNA sequence is then further processed into its final form by the ER and Golgi apparatus.

### Single nucleotide polymorphism and Genome Wide Association Studies

Although genes are inherited, the sequence of the genome as a whole is still unique in every individual. This is due to random changes in the nucleotide sequence: The most common occurrence is that one of the nucleotides in one DNA strand is substituted by another nucleotide in an event called single nucleotide polymorphism (SNP). SNPs are responsible for about 90% of the genetic variation between two individuals, equaling ca. 10 million SNPs in every individual's genome. A SNP located within a coding region of the genome may induce changes in the sequence leading to less expression of the corresponding gene, or a change of function of the protein formed. However, SNPs occurring in the coding region may not alter the amino acid sequence at all, in which case the SNP is said to be "silent". Further still, non-coding SNPs occurring outside of the genes may affect gene expression by introducing changes in nucleotide sequences in the vicinity of genes needed for promoters, silencers or enhancers<sup>139-141</sup>.

In 2007 the first large whole scan for T2D, the Diabetic Genetic Initiative (DGI), was performed in 1464 patients with T2D and 1467 matched controls from Scandinavia<sup>142</sup>. By performing such genome wide association studies (GWAs) it was possible to search for SNPs over the whole genome that associated with T2D, or at least with traits common in T2D such as high blood glucose, poor insulin response to glucose, or altered levels of hormones and incretins. The DGI helped to reveal several SNPs in genes that imposed an increased relative risk of T2D such as *TCF7L2*, *CDKAL1* and *KCNJ11*. The latter gene encodes, Kir6.2, the inward rectifier K<sup>+</sup>-channel in the K<sub>ATP</sub>-channel<sup>142</sup>. Interestingly, many of the SNPs identified were related to beta-cell function<sup>143-145</sup>. Association to T2D has also been shown for SNPs at the *KCNQ1* gene<sup>146</sup> encoding the voltage-gated K<sup>+</sup>-channel involved in action potential duration and frequency in INS-1 cells<sup>147</sup>.

### Changed levels of mRNA expression

Decreased mRNA levels of genes expressing proteins involved in exocytosis have been found in islets from human donors with T2D such as lower levels of *Stx1A*, *Stxbp1*, *SNAP25* and *VAMP2*<sup>22</sup>. The expression level of *Stxbp1* in beta-cells has also been found to be decreased in the diabetic Goto-Kakizaki rat-model (GK rat) when compared to the control Wistar rat-model<sup>148,149</sup>. Individual SNPs explaining the reduced mRNA levels

has proven difficult though. It may seem perplexing that while the genetic factor of inheritance for T2D is strong still no SNP has been found that exclusively pose an impact on the pathology of T2D. It is unlikely that any such single genetic change will be found because T2D is a complex disease influenced by both the environment and the genetic predisposition, and even more so, certain environments may influence individuals differently due to unique combinations of genetic properties in each individual<sup>150</sup>. Rather, a plethora of genetic changes may emerge that together exerts functional alterations thereby contributing to the inheritance of T2D.

### **Regulation by microRNA**

It has been shown that protein levels of *e.g.* Stxbp1 both in excess and recess reduce insulin secretion<sup>99</sup> which indicates that there is a window of expression in which optimal protein level need to be kept. As regulation of expression by insertion of SNPs is a random act, other factors likely contribute in maintaining intermediate protein levels. Protein-output can be regulated epigenetically by steps affecting expression of the gene<sup>151</sup>, but the gene transcript can also be modulated post-transcriptionally by microRNAs (miRNAs)<sup>152</sup>.

The miRNAs are small endogenously expressed RNA fragments that directly binds to the 3'UTR of their target mRNA. The primary transcript of miRNA (pri-miRNA) is generally transcribed by RNA polymerase II in the nucleus. The pri-miRNA contains a typical stem-loop structure that is processed by a nuclear enzyme complex including Drosha and Pasha, which releases a 60- to 110-nucleotide pre-miRNA hairpin precursor. The pre-miRNA is exported to the cytosol and further processed by the Dicer enzyme to yield the 19- to 22-nucleotide mature miRNA product. The mature miRNA is then incorporated into the RNA-induced silencing complex (RISC), which subsequently acts on its target by translational repression or mRNA cleavage<sup>153,154</sup>. As the miRNA does not require perfect complementary binding to the mRNA 3'UTR, multiple mRNAs may be targeted by a single miRNA and likewise, several miRNAs may target a single mRNA transcript.<sup>152</sup> In humans, miRNAs have been implicated in biological processes like cell proliferation and cell death during development, fat metabolism, insulin secretion, hematopoiesis and regulation of cell transformation<sup>155</sup>.

It is hypothesized that the level of mRNA encoding proteins involved in exocytosis may be regulated either directly or indirectly by miRNAs (miRNAs)<sup>156-158</sup>. In the context of T2D, several studies of miRNAs have emerged showing *e.g.* that miR-375 is up regulated in human T2D pancreatic islets<sup>159,160</sup> and overexpression of miR375 has been shown to regulate insulin secretion and exocytosis<sup>158</sup>, miR-96 and miR-124a reduce insulin secretion in MIN6B1 cells<sup>156</sup>, and the expression of miR-21, miR-34a and miR-146a is induced by IL-1 $\beta$  and TNF- $\alpha$  in human pancreatic islets<sup>161</sup>. In addition, 24 beta-cell specific miRNAs are up-regulated in the GK rat compared to the Wistar rat control<sup>162</sup>, amongst them miR-335.



# 4 Materials and Methods

## Cell culture

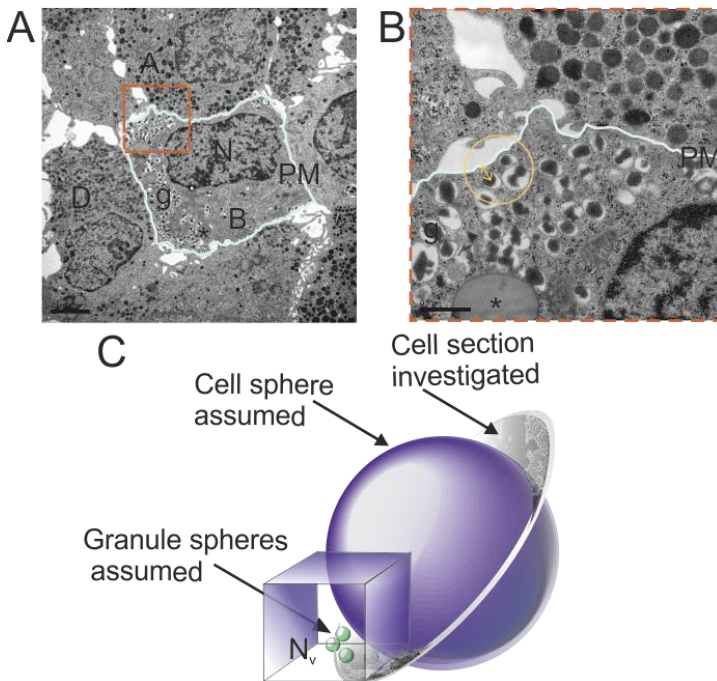
Several sources of cells have been used in the papers included in this thesis. Protein interaction and outcome on exocytosis are mechanistic events similar across many species meaning that these studies can well be performed in human islets, primary islets freshly extracted from mice, as well as in cell lines. In collaboration with Nordic Network of Islet Transplantation (Olle Korsgren at Uppsala University) and the human tissue laboratory at Lund University we have been granted islets from human diseased donors. The human islets are excellent tools to study events in human biology related to T2D however; the resources are scarce and dependent on human donors who have agreed to donation for research purposes. Primary cells have been extracted from the NMRI mouse strand and the advantage of freshly isolated cells is a closer resemblance to the *in vivo* situation and also, the cells have not undergone the genetic changes required to render cell-lines immortal. The disadvantage of primary cells is the limited lifespan, and restricted quantity. The cell-lines used in this thesis originate from either rat (INS-1 and INS1-832/13) or mice (MIN6) pancreatic insulinoma cells and thus share the properties of cancer-cells in that they continually divide. Cell-lines are preferred when a larger quantity of cells is needed, such as RNA-extraction. Hence, cell-lines spare the need to sacrifice several animals and furthermore, the inter-individual changes sometimes occurring in animals are limited by the use of cell-lines where all cells spring from the same origin.

## Transmission Electron Microscopy (TEM)

The maximum magnification that can be obtained using a conventional optical microscope is limited by the wavelengths of visible light, *i.e.* the relatively long wavelength of the photons enabling a resolution of  $\sim 200$  nm.

The transmission electron microscope (TEM) emits electrons instead of light. Electrons have much shorter wavelengths than photons thus enabling a larger magnification. The electrons travel through the microscope in vacuum and are focused into a very thin beam by electromagnetic lenses. When this beam travel through the slice of study, the electrons will either scatter of the slice and disappear from the beam, or continue through the slice and hit a fluorescent screen at the bottom of the microscope. This gives rise to a reflected image, with different nuances of darkness according to the different densities within. In order to visualize islets they need to be chemically fixed, dehydrated, embedded in plastic and cut into ultrathin slices with a thickness of  $\sim 90$  nm. Prefixation with glutaraldehyde was used to preserve the membranes.

Electron microscope images are highly defined allowing quantification of organelles such as lipid droplets, the total number of granules within the cell (Fig 5A), as well as the total number of granules docked at the plasma membrane. In this thesis, granules were defined as docked when the granule center was positioned within 150 nm from the membrane (Fig 5B). The different cell types were distinguished by means of granular appearance: Alpha-cells have small dense granules, beta-cells contain granules with a dense core surrounded by a white halo, and delta-cells have elongated less dense granules. However, as the image of the cell is a two-dimensional slice whereas the cell is in reality spherical, any quantification needs to conjure up into 3D (Fig 5C). Certain criteria are thus applied in order to transform the number of granules obtained in one section into an estimate of the total number of granules were the cell to be viewed in 3D. To create the 3D structure, I have calculated the Area ( $A$ ;  $\mu\text{m}^2$ ) and the perimeter ( $l$ ;  $\mu\text{m}$ ) of the cell in a randomly chosen slice in which I counted the total number of granules ( $N$ ) and the number of granules docked at the plasma membrane ( $N_{\text{dock}}$ ). Then the average diameter of each granule ( $d$ ;  $\mu\text{m}$ ) was calculated so as to determine the granule volume density ( $N_v$ ) of the total number of granules, and the surface density ( $N_s$ ) of the docked granules. Hence the formulas:  $N_v \approx (N/A)/d$  and  $N_s \approx (N_{\text{dock}}/l)/d$ . In order to not over- or under-estimate the total number of granules, only slices with a visual nuclear area  $\leq 1/3$  of the total cell area has been analyzed<sup>58,163</sup>.



**Fig 5** **A)** TEM image showing a delta-cell (D), alpha-cell (A) and beta-cell (B) with the plasma-membrane (PM) highlighted in blue surrounding the nucleus (N); granules (g), and lipid droplets (\*); **B)** Magnified TEM image highlighting in yellow a docked granule. The arrow indicate the measured distance between the PM and the center of the granule. The distance is defined as  $<150$  nm for the granules to be considered as docked. Scale bars:  $2 \mu\text{m}$  (A) and  $0.5 \mu\text{m}$  (B) **C)** Model describing the conversion of 2D parameters, derived from the image of the cell, into 3D parameters. Note that for purpose of illustration, the perimeter of the cell section is magnified

The 3D diameter of the granules cannot be readily solved by means of measuring the width of each granule only, because the slice is ultrathin (70-90 nm) and the granules are differently positioned within the cell, meaning that each granule will be cut at different positions. This has been solved by Giger and Riedwyl in 1970<sup>163</sup> roughly: Diameter data from a large number of cells and grids is collected and the mean granule diameter is calculated ( $d$ ), which needs to be multiplied by  $4/\pi$  to derive a first estimate ( $D_1$ ). The frequency of the diameters sizes are then plotted to control for a Gaussian distribution. From this histogram the fraction( $Q$ ) of granules with a diameter  $>D_1$  is estimated and used to get a value  $F(Q)$ <sup>163</sup>. The real diameter  $D_2$  can thus be determined as  $F(Q)*d$ .

### **Confocal Laser Microscopy**

For the purpose of studying proteins such as SNAP25 and Stx1A, confocal fluorescence microscopy has been used which enables fluorescently tagged proteins to be visualized by utilizing a laser beam. In confocal laser microscopy photons are used as light source, but processed so that rather than being scattered the photons are focused along a single wavelength, the laser beam. Each fluorescent tag emits photons at specific wavelengths upon excitation by the laser beam and the different light emissions is collected and presented as images in a computer. The confocal microscope has the great advantage for interior imaging because it focuses the beam onto specific depths. Hence, the laser beam can “pin point” the focus through the cell one layer at a time; the layers can later be superimposed on top of each other in the computer which allows for a 3D-visualization.

### **Patch-Clamp and capacitance measurements**

The Patch-Clamp technique has the great advantage that it allows measurements on living single cells with a high temporal resolution. The method to measure electrophysiological activity of a cell was first developed by Alan Lloyd Hodgkin and Andrew Huxley for which they were rewarded the Nobel Prize in Physiology and Medicine in 1963, a prize also awarded in 1991 to Bert Sakmann and Erwin Neher who refined the electrophysiological technique by the invention of the patch-clamp technique<sup>164</sup>.

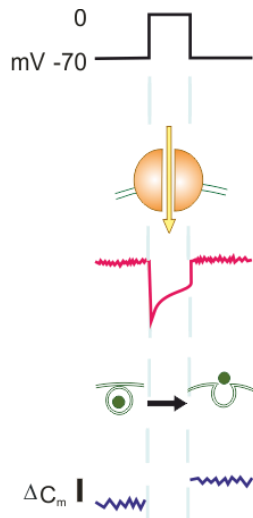
The patch-clamp technique allows clamping the cell to a fixed membrane potential and measure ion channel currents. This is achieved by formation of a high resistance gigaohm-seal between the cell membrane surface and a small, glass capillary tube filled with pipette solution surrounding a chlorided silver electrode connected to an amplifier. The gigaseal isolate the membrane patch electrically, minimizing any leak currents. At this point, several configurations can be achieved: In this thesis I have used the standard whole-cell configuration where the small patch of membrane within the pipette tip opening is ruptured, whereby equilibrium between the pipette solution and the cell interior is reached. This setting allows measuring the sum of currents from all ion channels within the cell. Clamping of the cell membrane potential is achieved by the generation of a feedback current that compensates for the currents created over the membrane. The potential of the cell-membrane is constantly detected and compared

with the potential demanded: Any differences between the dictated potential and the actual potential measured by the patch-clamp amplifier generate an injection of compensatory current. The size of the current needed to compensate for alterations in the membrane potential is recorded onto the computer screen.

The standard whole-cell configuration can also be used to measure exocytosis by means of cell membrane capacitance. The cell membrane separating two electrically charged fields equals a capacitor. The capacity to store charge (Capacitance; measured in Farad) by any given capacitor depends on factors summed up in the equation

$$C = \epsilon_r \epsilon_o (A/d)$$

where the cell capacitance (C) properties are defined as follow: ( $\epsilon_r$ ) is the material constant of the phospholipids times the permittivity constant ( $\epsilon_o$ ), (A) equals the surface area of the cell which is divided by the distance (d) between the two layers of phospholipids. As the ( $\epsilon_r$ ,  $\epsilon_o$  and d) of the cell membrane are constant factors, only the area and capacitance varies upon fusion of granules with the plasma membrane;  $C=A$ . Thus, during exocytosis the incorporation of granular membranes into the cellular plasma membrane gives that the area of the plasma membrane increases and hitherto, the capacitance increase. Therefore, it is possible to measure exocytosis by the increase in cell capacitance. Exocytosis evoked by artificial depolarizations of the membrane potential will activate voltage-dependent  $Ca^{2+}$ -channels thereby creating a current of  $Ca^{2+}$ -influx which will in turn provoke exocytosis and as a consequence, cell membrane capacitance increase (Fig 6).



**Fig 6** Illustration of depolarization-evoked exocytosis

## Quantitative RT-PCR (qRT-PCR)

RNA extracted from cells is amplified into its complementary DNA (cDNA) strand by polymerase chain reaction (PCR). The first step is the reverse transcription of mRNA into cDNA using random primers, or stem-loop primers for miRNA. The second step, qRT-PCR is then performed using gene-specific primers and TaqMan probes.

In the qRT-PCR step, primers and probes specific for each gene of interest are used in the reaction. The TaqMan-based qRT-PCR is generally a 5' nuclease assay, wherein a FAM dye reporter bound to the oligonucleotide probe is released at each amplification step. During the exponential phase of amplification, the emitted fluorescent signal is directly proportional to the amount of target cDNA to which the probes are hybridized. The threshold cycle (CT) for the subsequent proportional increase in fluorescence per qRT-PCR cycle is used to quantify the amount of cDNA being amplified. Rapid increase in fluorescence corresponding to low CT-values indicates a large quantity of cDNA templates in the first cycle. However, it is vital to compare the fluorescence of the gene of interest individually. Since the amplification step in the qRT-PCR is so sensitive, subtle differences between the amounts of starting material (DNA-input) or quality of the cDNA in the first cycle would be highly magnified. To correct for this, genes known to be stably expressed regardless of treatment status are also detected in the qRT-PCR. This normalization procedure is commonly called the  $\Delta\Delta C_t$ -method where the CT for the gene expression of interest is divided by the CT of the stably-expressed gene.

## Statistics

### *Statistical analyzes used in DGI and GWA*

In the case of investigating mRNA levels of exocytotic genes (paper III) the  $\chi^2$ -test was applied, followed by functional studies on all genes found nominally significantly altered to additionally exclude the possibility of false positives. The  $\chi^2$ -test can be utilized when it is of interest to compare if multiple findings differ significantly from a predicted outcome. Roughly, in this test the expected rate of random associations with *e.g.* T2D in a large data set is first calculated, and then the number of actual associations with T2D in a selected group of genes is observed, and divided with the expected number. If there is less than 5% chance that an observed number found is due to random changes, then the associations detected in the data sheet are considered *nominally* significant. A  $\chi^2$ -test does not penetrate individual differences but gives an estimate if a certain number of observed changes exceed the number of changes that would be statistically expected.

In the investigation of SNPs association to T2D (paper III) a “functional biological” approach have been applied by selecting genes coding for proteins used in exocytosis. Further, SNPs were only investigated for association with phenotypes that would be expected as a result of interfered functional exocytosis. By also requiring that a SNP need to associate with at least two phenotypes simultaneously, each with significant levels below 0, 05%, it was hypothesized to reduce the likelihood that SNPs meeting these multiple criteria was a random false positive finding.

A *significant* association of a SNP to T2D is less than 5% likely to be due to random alterations in a normally distributed population. That is, a small uncertainty in association is accepted because it is 95% certain that the finding is true, and not a false positive association with T2D. However, when multiple factors are correlated, the random chance that one of the factors will appear more commonly in individuals with T2D increases proportionally. To counteract for large scale comparisons the Bonferroni correction can be applied. Roughly, this is a way to punish a large dataset by dividing the significance level with the number of tests performed. However, at the point of very large data sets the Bonferroni correction may instead create the possibility of false negatives. That is, there may be SNPs in the data sheet that have a true association with T2D but due to the hard restrictions applied these fail to meet the new criteria and hence, goes by unnoticed.

#### *Association Studies- Relative Risk*

Association studies are generally performed comparing two groups being as identical as possible in terms of age, weight, health history etc., but in which one group has a possible risk factor; in this case T2D (paper IV). All genetic changes in the two groups are then detected whereby the changes can be associated with the number of individuals that develop T2D. The breakthrough of T2D genetics came in 2006 with the identification of a genetic variant in the gene encoding the transcription factor TCF7L2. TcF7L2 is important for transcription of genes involved in the WNT-pathway and the SNP in the *TCF7L2* gene associated with a 1.4 times higher relative risk of developing T2D<sup>165</sup>. In other words, T2D occurred in both groups but there was an *additional* risk of 1.4 to develop T2D in individuals with the genetic change in TCF7L2 (or rather backwards, individuals in the risk group developing T2D had a 1.4 times higher occurrence of the genetic change in TCF7L2). Therefore, a mutation conferring an increased relative risk is not a tool to predict if an individual will develop a certain disease, because not all individuals carrying the genetic change do so. It is to be viewed as an indication that said persons has a 1.4 higher risk of developing T2D as compared to the *general* risk of developing T2D in individuals that does not carry the mutation.

#### *Statistical analysis of calcium sensitivity*

In the context of the investigation of Ca<sup>2+</sup>-sensitivity in paper II we used a mixed effect model instead of a linear regression model. Mixed-effects models<sup>166</sup> cope with random variation due to biological differences between cells. We assumed a fixed effect of Ca<sup>2+</sup> on exocytosis thereby quantifying the average Ca<sup>2+</sup>-efficacy within each group. Then between-cell variation in Ca<sup>2+</sup>-efficacy (random effect) was modeled by a zero-mean normal distribution with an estimated standard deviation testing whether the treatment differed from zero. Next, we imposed the random effect to adjust the model for deviations in individual cell responses from the group average. This analysis enables to statistically test whether treatment (in paper II with anti-Stx1A) alters the efficacy of Ca<sup>2+</sup> on exocytosis.

# 5 Aims of the present investigation

The general objective of this thesis was to elucidate factors influencing exocytosis in the pancreatic islet cells.

The specific aims were to:

- I. Examine the role of SNAP25 in cAMP-enhanced exocytosis and PKA-independent priming in insulin secreting cells.
- II. Investigate if localization of SNAP25 and Stx1A within the mouse alpha-cell is glucose-dependent, and how these proteins influence exocytosis.
- III. Explore whether altered expression of genes encoding proteins involved in exocytosis correlate with glucose homeostasis and T2D in human donors.
- IV. Investigate whether T2D genetic risk variants associate with altered granular docking, exocytosis and insulin secretion in islets from human donors.
- V. Study whether miR-335 modifies the expression of Stxbp1 and SNAP25 and alters exocytosis in INS1-832/13 cells.

# 6 Results and Discussion

## Paper I

### **Truncation of SNAP25 reduces the stimulatory action of cAMP on rapid exocytosis in insulin-secreting cells**

It is well known that incretins such as GLP-1 stimulate exocytosis in the beta-cell. Incretin stimulation gives rise to elevated levels of intracellular cAMP, which in turn activates second messenger pathways that potentiate the effects of  $\text{Ca}^{2+}$  influx. The central function of cAMP is to activate the enzyme PKA. However, cAMP also affects exocytosis via PKA-independent mechanisms such as directly interacting with cAMP-GEFII; a protein that in beta-cell exocytosis is involved in granular priming. We aim to explore the mechanism by which SNAP25 is involved in cAMP-dependent exocytosis and specifically evaluate if SNAP-25 is important for PKA-independent priming of insulin containing granules.

#### **Both full-length and truncated SNAP25 binds to cAMP-GEFII and Rim2**

PKA-independent stimulation of exocytosis involves a complex formation in which cAMP-GEFII interacts with several proteins including SUR1 and Rim2. Using a GST-binding assay, full-length SNAP25 (SNAP25<sub>WT</sub>) and a truncated C-terminal form of SNAP25 (SNAP25<sub>1-197</sub>) were found to bind equally to both Rims2 and cAMP-GEFII, suggesting that the binding domains are not located at the C-terminal part of SNAP25.

#### **Truncation of SNAP25 reduce cAMP-dependent rapid exocytosis**

To further evaluate the role of SNAP25 in cAMP-stimulated exocytosis, capacitance measurements were performed on single INS-1 cells overexpressing SNAP25<sub>WT</sub>, SNAP25<sub>1-197</sub> or Botulinum neurotoxin A (BoNT/A). The latter cleaves SNAP25 at the C-terminal removing the nine last amino acids. To investigate effects on rapid exocytosis of granules within the immediately releasable pool (IRP), a subpopulation of the RRP, capacitance measurements was performed. Changes in membrane capacitance was evoked by the application of membrane depolarizations (from -70 mV to 0 mV) with increasing pulse duration from 5-450 ms allowing an increased amount of  $\text{Ca}^{2+}$  to enter. The achieved increase in membrane capacitance reached a plateau at the longer depolarizations and a mathematical model describing the release kinetics when granules move from IRP to a fused state was used to estimate the size of IRP. Intracellular application of cAMP in the patch-pipette increased the release from IRP almost 4-fold in INS-1 cells overexpressing SNAP25<sub>WT</sub>, similar to the situation in non-transfected cells. In cells expressing the truncated form of SNAP25 or BoNT/A, cAMP



failed to increase rapid exocytosis. However, this could be overcome by intracellular application of full-length SNAP25 through the patch pipette.

In order to investigate the role of SNAP25 on cAMP-dependent refilling of RRP, we applied another protocol where exocytosis was evoked by a train of ten 500-ms depolarizations from -70 mV to 0 mV. The ability of cAMP to enhance the exocytotic response to the first two depolarizations of the train, representing a maximum estimate of the RRP, was significantly reduced in cells expressing SNAP25<sup>1-197</sup> compared to cells overexpressing SNAP25<sup>WT</sup>. The ability for cAMP to increase granular refilling, represented by the increase in membrane capacitance during the latter 8 depolarizations, was unchanged in SNAP25<sup>WT</sup> - and SNAP25<sup>1-197</sup>- expressing cells. Together these results indicate that the transducing domain of SNAP25 on cAMP-mediated exocytosis is at the C-terminal of the protein and that SNAP25 influences the actions of cAMP on IRP/RRP release rather than affect the refilling of granules from the RP.

#### **cAMP-enhanced GSIS is not altered in SNAP25<sup>1-197</sup>-expressing cells**

We were interested in whether the effects of SNAP25 modulation observed in the exocytotic responses could be transferred to similar responses in cAMP-potentiated GSIS in INS-1 cells overexpressing SNAP25<sup>WT</sup> and SNAP25<sup>1-197</sup>. To be able to measure insulin secretion only from transfected cells we made use of a Growth Hormone (GH) assay, where the SNAP25 constructs were co-transfected with a vector overexpressing GH. Cells transfected with an empty vector together with the GH was used as controls. Transfected cells were incubated for 1 hour in medium containing 20 mM glucose in absence or presence of forskolin and IBMX. Addition of forskolin and IBMX caused a 3-4-fold increase in GSIS in both SNAP25<sup>WT</sup> and SNAP25<sup>1-197</sup>-overexpressing cells as compared to control. This data are in agreement with the results from the capacitance measurements where refilling was investigated using a train of membrane depolarizations.

In summary:

- 1) SNAP25 binds to both cAMP-GEFII and Rim2 towards the N-terminal
- 2) SNAP25 is essential for cAMP-induced rapid exocytosis, mediated via the C-terminus

## Discussion paper I

The cAMP-enhanced exocytosis of granules belonging to IRP/RRP does not increase upon overexpression of SNAP25<sub>WT</sub> in INS-1 cells indicating that the endogenous level of SNAP25 is already at a saturating capacity to stimulate exocytosis through cAMP. This component of exocytosis has been attributed to be mediated through cAMP-dependent activation of cAMP-GEFII<sup>76</sup>. SNAP25 may contribute to the cAMP-dependent rapid exocytosis because SNAP25<sub>WT</sub> was found to bind to cAMP-GEFII and Rim2. This binding most likely occurs at the N-terminal of SNAP25 as SNAP25<sub>1-197</sub>, truncated at the C-terminal, also successfully bind to cAMP-GEFII. However, maximal exocytosis of IRP/RRP granules is still reduced in INS-1 cells overexpressing SNAP25<sub>1-197</sub> or BoNT/A, thus suggesting that the C-terminal part of SNAP25 is important for transducing the cAMP-enhancing signal on rapid exocytosis.

Refilling of granules from the RP to the RRP is a PKA-dependent process<sup>73</sup> where PKA has been found to phosphorylate SNAP25 at Thr<sub>-138</sub><sup>167</sup>. This site thus remains intact in SNAP25<sub>1-197</sub> which could explain why only the rapid, but not the slow, exocytosis is affected in the INS-1 cells overexpressing SNAP25<sub>1-197</sub>. Insulin secretion measurements over 1 hour support this observation in that cAMP-potentiated GSIS did not differ between cells overexpressing SNAP25<sub>WT</sub> and SNAP25<sub>1-197</sub>.

We propose that the C-terminal of SNAP25 mediates cAMP-enhanced release of IRP/RRP without affecting the refilling of granules from the RP. However, the finding that cAMP-GEFII and Rim2 bind to SNAP25 suggests that the N-terminal could also contribute in the cAMP-dependent rapid exocytosis. These insights are of specific relevance knowing that the first phase insulin secretion associated with release of IRP/RRP granules is reduced in T2D. In this context it is also of interest that the level of SNAP25 is reduced in human islets from donors with T2D<sup>22</sup> and reduced expression of SNAP25 in human islets associate with increased HbA1c (paper III).

## Paper II

### **Glucose-dependent docking and SNARE protein-mediated exocytosis in mouse pancreatic alpha-cell**

A typical feature of T2D is a disturbed alpha-cell function where glucagon secretion is increased at hyperglycemia but decreased at hypoglycemia. Stx1A and SNAP25 are well established as crucial for beta-cell exocytosis, but less is known about the performance of these proteins in alpha-cell exocytosis. In this study we aimed to investigate the temporal role of Stx1A and SNAP25 in the exocytotic process of mouse alpha-cells.

#### **Glucose-dependent localization of SNAP25 and Stx1A in mouse alpha-cells**

First, we used confocal microscopy to investigate whether the intracellular distribution of Stx1A and SNAP25 is glucose-dependent. Primary mouse islet cells were extracted and subjected to low (2.8 mM), medium (8.3 mM) and high (16.7 mM) glucose incubation previous to performing immunohistochemical labelling of Stx1A, SNAP25 and glucagon. The distribution was quantified by calculating a ratio of fluorescence intensity in the vicinity of the plasma membrane (0.5  $\mu\text{m}$ ) to that in the intracellular region (sans nuclear area). SNAP25 localisation in the plasma membrane was highest after incubation in low glucose and redistributed to the cytoplasm following medium and high glucose stimulation. Stx1A resided primarily in the cytosol at low and high glucose but tended to re-distribute to the membrane at intermediate glucose levels. As SNAP25 and Stx1A are part of the SNARE-complex, we also measured the co-localisation between SNAP25 and Stx1A. This analysis reveal that  $\sim 40\%$  of the Stx1A molecules co-localise with SNAP25 following incubation in low glucose which increases to  $\sim 53\%$  and  $\sim 70\%$  following medium and high glucose stimulation, respectively.

#### **Antibodies against SNAP25 reduce exocytosis of glucagon-containing granules**

To study the effect of SNAP25 proteins on glucagon exocytosis, single mouse alpha-cells were stimulated by a train of depolarizations using the standard whole-cell configuration of the patch-clamp technique in combination with capacitance measurements, where antibodies against SNAP25 was added intracellularly via the patch-pipette. An IgG antibody used in control experiments ascertained that the anti-SNAP25 antibody did not reduce exocytosis by steric hindrance. Anti-SNAP25 addition reduced the total exocytotic response by  $\sim 44\%$ . Closer inspection of the pulses revealed that the reduction was mainly exerted at the later phase corresponding to refilling of the RRP by granules from the RP, causing a  $\sim 70\%$  reduction in the capacitance increase. The rapid exocytosis of granules from the RRP was also hampered, albeit to a lower extent ( $\sim 45\%$ ). Thus, SNAP25 interfere in the exocytotic process releasing granules of both the RRP and the RP.

### **Alpha-cell exocytosis is reduced in presence of antibodies against Stx1A**

Using the same protocol as described, exocytosis was studied when antibodies against Stx1A was applied. The total exocytotic response was reduced by ~50% upon addition of anti-Stx1A. This was reflected by a ~50% reduction of granules belonging to the RRP as well as ~30% decrease in the refilling of the RRP. To closer evaluate the impact of Stx1A in the rapid exocytosis of RRP we applied a protocol with depolarizations of increasing pulse duration from 5-850 ms. Measurable exocytosis could be detected already after 10 ms albeit anti-Stx1A inclusion did not exert a significant reduction in capacitance below 100 ms. Hence, both rapid release from the RRP and refilling of the granules was significantly repressed by inclusion of anti-Stx1A.

### **Antibody against Stx 1A is not associated with a reduced Ca<sup>2+</sup>-current**

Reduced exocytosis following antibody inhibition of Stx1A in the mouse beta-cell has been explained by a concomitant reduction of the Ca<sup>2+</sup>-current. To investigate this relationship in the mouse alpha-cell we applied 50 ms depolarizations ranging from -70 mV to between -40 mV and +40 mV thereby eliciting voltage-dependent currents. Unlike the beta-cells, anti-Stx1A treatment did not impair the Ca<sup>2+</sup>-current. To measure the Ca<sup>2+</sup> sensitivity, the exocytotic capacity at any given Ca<sup>2+</sup>-concentration was investigated by calculating the slope between increases in membrane capacitance against the integrated Ca<sup>2+</sup>-current in the rapid exocytosis during pulse durations ranging between 5-850 ms. To account for cell-to-cell differences, we applied the mixed-effect statistical model where we considered a fixed effect of Ca<sup>2+</sup> on exocytosis and included a random effect to adjust the model for how the individual cells responses deviated from the group estimate. When no antibody was included, the Ca<sup>2+</sup> sensitivity was estimated to ~18 fF/pC, which was significantly reduced to ~10 fF/pC following addition of anti-Stx1A.

### **Increased glucose associate with increased number of docked granules**

We proceeded to study the granule localization using EM following incubation in low, medium and high glucose (1.0, 8.3 and 16.7 mM glucose, respectively). To estimate the number of docked granules, the surface density of granules situated within 150 nm from the plasma membrane was calculated. At high glucose stimulation the numbers of docked granules increased two-fold as oppose to low glucose. The total number of granules (~4000) remained static independently of preceding glucose-incubation protocol and likewise, the granule diameter of ~275 nm did not change.

In summary:

- 1) SNAP25 localizes to the plasma membrane at high glucose, and antibody inhibition of SNAP25 reduces exocytosis
- 2) Stx1A co-localization with SNAP25 increases at high glucose, and anti- Stx1A reduce exocytosis but not the Ca<sup>2+</sup> current, albeit the Ca<sup>2+</sup> sensitivity decrease.
- 3) The number of docked granules in the alpha-cell increase at high glucose, without affecting the diameter or the total number of granules

## Discussion paper II

We hypothesize that redistribution of SNAP25 from the plasma membrane to the cytosol in alpha-cells may contribute to decreased glucagon secretion as SNAP25 relocalisation in mouse beta-cells has been shown to decrease exocytosis and insulin secretion<sup>89</sup>. Syntaxin molecules have been shown to diffuse away from the site of fusion following exocytosis<sup>60</sup> which may contribute to the accumulation of granules near the plasma membrane at higher glucose concentrations. It is suggested that Stxbp1 participate in the transport of Stx1A from the ER and Golgi region to the plasma membrane<sup>88</sup>. PKC has been shown to regulate alpha-cell exocytosis<sup>168</sup> and phosphorylate Stxbp1<sup>169</sup> which indicate that PKC participate in the transport of Stx1A.

The SNARE complex are in a tight conformation when primed granules are ready for release upon  $\text{Ca}^{2+}$ -influx which could explain why the anti-Stx1A antibody does not reduce exocytosis below 100 ms. Stx1A has been found to affect the R-type  $\text{Ca}^{2+}$ -channels in the presence of cAMP in the beta-cell, whereas exocytosis in the alpha-cell is mainly dependent on influx through the L-type  $\text{Ca}^{2+}$ -channels which could explain why we could not detect reduced  $\text{Ca}^{2+}$ -current upon anti-Stx1A inclusion in the alpha-cells. However, the reduced  $\text{Ca}^{2+}$ -sensitivity of exocytosis following anti-Stx1A addition suggests that Stx1A attributes a direct effect on the exocytotic machinery. This could be due to impaired binding to the  $\text{Ca}^{2+}$ -sensing  $\text{C}_2\text{A}$  domain of Syt7<sup>133</sup>; a protein suggested being the major  $\text{Ca}^{2+}$ -sensor of  $\text{Ca}^{2+}$ -dependent exocytosis in the alpha-cell<sup>134</sup>.

Our ultra-structural analysis reveal that ~140 granules remains docked at 1 mM glucose indicating that part of this docked pool consists of unprimed granules as the pool of docked granules is not emptied during the maximal alpha-cell stimulation. Thus, the decreased fluorescence of Stx1A and SNAP25 at the plasma membrane in high glucose does not necessarily mirror the increased docking but perhaps rather the reduction in exocytosis of these granules. Following high glucose stimulation the pool of docked granules increases to ~310, out of which only ~50 granules can be accounted for by reduced exocytosis. The remaining increase is most likely due to increased rate of docking. The reduced secretion might partly be due to reduced presence of SNAP25 at the plasma membrane, and the increased docking is most likely a result from increased mobilization caused by the increased concentration of ATP at higher glucose concentrations. In summary, the increased number of docked granules at 16.7 mM glucose is a combination of reduced secretion and increased docking where the majority (70%) is a result of the increased docking.

In conclusion, we show that the localization of Stx1A and SNAP25 is glucose dependent and that interferences with these proteins influence temporal aspects of exocytotic process in the alpha-cell. Neither antibody inhibition of Stx1A nor SNAP25 reduce the voltage-dependent  $\text{Ca}^{2+}$ -current, suggesting that these SNARE proteins regulate fusion as part of the exocytotic machinery, rather than through modulation of the  $\text{Ca}^{2+}$ -channel influx. Moreover, we estimate the number of granules in the mouse alpha-cell to be ~4000 and demonstrate that granule docking in the alpha-cell is glucose-dependent.

## Paper III

### **Reduced insulin secretion in human Type 2 diabetes correlates with decreased expression of exocytotic genes**

Successful beta-cell exocytosis involves the interaction of several proteins such as SNAP25, Stx1A, VAMP2, Stxbp1 and the family of Syts. A microarray investigating the expression of 21 000 genes in human pancreatic islets from 55 non-T2D and 9 T2D deceased Scandinavian donors revealed that the expression of 1300 genes were down-regulated in individuals with T2D. We compared the mRNA expression of 23 genes encoding proteins involved in exocytosis on the microarray between human donors with and without T2D.

#### **Decreased expression of exocytotic genes in human islets from T2D donors**

Using Mann-Whitney U-test we compared the expression levels of 23 exocytotic genes between 9 islets from donors with T2D with 55 islets of non-T2D origin (ND). We found that the expression levels of the genes encoding *STX1A*, *SYT4*, *SYT7*, *SYT11* and *SYT13* is nominally down-regulated in the islets from donors with T2D. A  $\chi^2$ -test performed endorse that the observed number of genes involved in the exocytotic process being down-regulated was significantly higher ( $p < 0.05$ ) than could be expected by random changes. Further, we technically replicated the down-regulation found in the microarray with qRT-PCR ensuing the decreased gene expression of *STX1A*, *SYT4* and *SYT7* in islets from donors with T2D ( $n=7$ ) as compared to non-T2D donors ( $n=47$ ). The reduced expression was accompanied by reduced protein levels of STX1A, SYT4 and SYT11 in islets from T2D donors ( $N=2$  ND and  $N=1-3$  T2D donor).

#### **Correlation of gene expression involved in exocytosis to GSIS and HbA1c**

Spearman correlation analysis revealed that the expression levels of *STX1A*, *SYT4*, *SYT7*, *SYT11* and *SYT13* correlated negatively to measurements of HbA1c *in vivo*. The expression was concomitantly correlated positively to GSIS measurements *in vitro*. In addition, the expression levels of *SNAP25* and *STXBP1* was also found to associate negatively to HbA1c levels ( $p=0.018$ ,  $0.007$ , respectively) and positively to GSIS ( $p=0.042$ ,  $0.038$ , respectively). None of the expression levels correlated to basal insulin secretion (*i.e.* insulin secretion at 1 mM glucose), which indicates that the Ca<sup>2+</sup>-dependent exocytosis is affected, rather than the basal insulin secretion.

#### **Functional effects on insulin secretion**

To investigate the functional effects of reduced expression of exocytotic genes we performed insulin secretion measurements following siRNA silencing of these genes in INS1-832/13 cells. SiRNA silencing of *Syt4*, *Syt7* and *Syt13* significantly reduced GSIS, whereas there was no decrease in insulin secretion due to silencing of *Syt11* and *Stx1A*. Instead, *Stx1A* silencing increased the secretory response. This may be a reflection of

that *Stx1A* has both inhibitory and stimulatory function. Indeed, also overexpression of *Stx1A* has proven to be negative for insulin secretion in rodents<sup>92</sup>.

### **Polymorphisms in the *RIMS1* gene associate with impaired insulin secretion**

Using the GWA DGI we studied the occurrence of SNPs in or near the 23 exocytotic genes that associate with reduced gene expression. To compensate for the multiple associations performed we restricted the study to that a SNP must display a nominal significant association (requiring p-values  $\leq 0.01$  and Minor Allele Frequency  $\geq 0.05$ ) with at least 2 phenotypic traits related to altered exocytosis namely fasting insulin, 2-h insulin, fasting glucose, 2-h glucose and/or insulinogenic index. The top candidate SNPs, rs12524194 and rs1339226 in *RIMS1* were associated with 2-h glucose and insulinogenic index with positive beta-values for both phenotypes. The positive beta-coefficient for insulinogenic index indicates that the major allele associate with reduced insulin secretion. However, these associations could not be replicated in the PPP-Botnia study but the G-allele of rs12524194 associate with reduced expression of *RIMS1* (p=0.045) in human islets from ND donors.

In summary:

- 1) The expression of *STX1A*, *SYT4*, *SYT7*, *SYT11* and *SYT13* is reduced in human pancreatic islet cells of donors with T2D
- 2) The expression of *STX1A*, *SYT4*, *SYT7*, *SYT11* and *SYT13* correlates negatively with HbA1c and positively with GSIS, but basal insulin secretion is not affected. A finding also true for *SNAP25* and *STXBP1*.
- 3) Insulin secretion is reduced in INS1-832/13 cells upon silencing of *Syt4*, *Syt7* and *Syt13*
- 4) The SNP rs12524194 correlated with reduced gene expression of *RIMS1* and with 2 hour glucose and insulinogenic index

### Discussion paper III

We hypothesize that the expression of exocytotic genes is altered in islets from donors with T2D, which could contribute to the loss of first phase insulin secretion typically seen in T2D patients.

Compared to a previous study by Östenson et al., (2006) we could confirm the down-regulation of the *STX1A*, *SNAP25* and *STXBP1* gene expression but not of *SYT5* or *VAMP2* in islets from donors with T2D or high HbA1c. Furthermore, we identified several novel members belonging to the SYT-family *i.e.* *SYT4*, *7*, *11* and *13* to be down-regulated in islets from T2D donors. Regardless of the disparities it highlights that reduced expression of exocytotic genes are involved in T2D. It is of interest that *SYT11* has not previously been shown to be expressed in human pancreatic islets.

The expression of exocytotic genes appear to mainly affect the Ca<sup>2+</sup>-dependent exocytosis as no correlations were detected between the expression levels and basal insulin secretion (when Ca<sup>2+</sup>-concentration in the cell is low due to inhibited Ca<sup>2+</sup>-influx). This is in line with our finding that the expression of several SYTs suggested to act as the main Ca<sup>2+</sup>-sensors of exocytosis are down-regulated. We speculate that reduced expression of exocytotic genes in islets affects blood glucose levels through impaired insulin secretion which is reflected by the concomitant negative correlation with HbA1c and positive correlation to GSIS. Östenson et al., (2006) demonstrated that reduced expression of exocytotic genes translate into reduced protein levels and likewise, we confirm by confocal microscopy that Stx1A protein level is reduced and further, by Western Blot analysis that SYT4 and SYT11 protein levels are decreased in human islets from donors with T2D.

Given the rather extensive SNP analysis on the 23 exocytotic genes performed, we are tempted to conclude that SNPs in these genes is not the primary cause of impaired expression. However, two studies have previously shown SNPs in Stx1A T2D<sup>170,171</sup> and it is possible that if we were to conduct an even larger study additional SNP associations could be detected. Epigenetic factors including DNA methylation and histone modifications have been found to decrease gene expression related to reduced insulin secretion<sup>145</sup> and further expression of Snap25, Stx1A and Vamp2 has been shown to be regulated via the PI3K/Akt pathway and FoxO1 in mouse<sup>172</sup>. Down-regulation of exocytotic genes may also be attributed to the action of miRNAs<sup>173</sup>. We have recently detected an up-regulation of rno-miR-335 in the diabetic GK-rat model which target Stxbp1<sup>162</sup> and that decreased levels of rno-miR-335 associate with increased insulin secretion (paper V). In this context, it is interesting that we found that the expression of *STXBP1* in human islets positively correlated to GSIS although we could not detect any SNPs that associated with reduced expression of these genes (data not shown).

We conclude that the gene expression of key players involved in the exocytotic process is altered in islets from T2D donors. Additional studies are needed to evaluate if these alterations are translated into functional effects, especially to unravel whether down-regulations of these genes are a cause or effect of reduced insulin secretion in the development of T2D.



## Paper IV

### Reduced insulin exocytosis in human pancreatic beta-cells with gene variants linked to type-2 diabetes

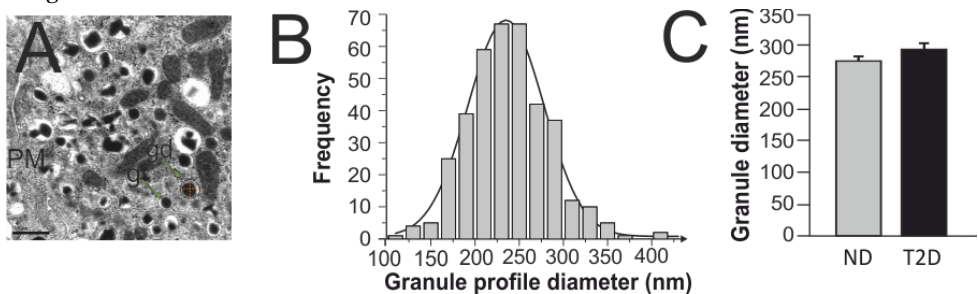
Recent GWA have identified a large number of loci associated with an increased risk of T2D, of which many are associated with reduced insulin secretion. In this study we aimed to investigate exocytosis and ultra-structural parameters in human islets from ND and T2D donors and to identify genetic variants of T2D-associated alleles that influence beta-cell exocytosis, granular density and docking.

#### GSIS, but not exocytosis, is reduced in islets from donors with T2D

GSIS increased ~5-fold in islets from ND donors, this fold-increase was reduced by 50% in islets from donors with T2D. However, capacitance measurements evoked by a train of 10 depolarizations showed no significant difference in the exocytotic response between the two groups.

#### Unaltered number of granules, docked granules and granule diameter in T2D

Electron micrographs were taken on human islets and granular diameter, granular volume density ( $N_v$ ; measure of total number of granules), granular surface ( $N_s$ ; measure of docked granules) and number of lipid droplets and lipofuscin bodies were estimated. The granular diameter was estimated to be  $275 \pm 7$  nm ( $n=14$  donors) in beta-cells from ND donors and  $294 \pm 10$  nm ( $n=8$  donors) in beta-cells from T2D donors (Fig 7). Thus, there was no significant difference in the granular diameter between ND and T2D. Likewise, there was no significant difference between the two groups when comparing  $N_v$  and  $N_s$ , which was estimated to  $4.7 \pm 0.3$  granules/ $\mu\text{m}^3$  and  $0.30 \pm 0.04$  granules/ $\mu\text{m}^2$  in ND donors and  $4.9 \pm 0.3$  granules/ $\mu\text{m}^3$  and  $0.26 \pm 0.03$  granules/ $\mu\text{m}^2$  in beta-cells from T2D donors, respectively. However, we found a strong correlation between BMI and the number of lipid droplets and in agreement with a previous investigation we also could confirm that the number of lipofuscin bodies increased with the age of the donor.



**Fig 7** **A)** Electromicrograph of a beta-cell, plasma membrane (PM), granules (g) are indicated as well as the measure of granule profile diameter (gd) **B)** Histogram of profile granule diameter distribution **C)** Bar diagram of average granule diameter in human beta-cell from ND and T2D donors.

## Genetic variants associate with reduced beta-cell exocytosis and granular docking

A multitude of genetic factors likely contribute to T2D and therefore 14 SNPs were selected based on their association with reduced insulin secretion *in vivo* in already published studies<sup>79,142,146,174,175</sup>. Of these, 4 SNPs were found to be associated with the exocytotic response. Exocytosis was reduced in *TCF7L2* rs7903146 risk allele carriers, in carriers of the *KCNJ11* rs5219 T allele, in islets from non-obese donors carrying the *ADRA2A* rs553668 risk allele and finally, rs2237895 located in intron 15 of the *KCNQ1* gene. For the latter, exocytosis improved upon addition of the antagonist linopirdine against the K<sup>+</sup> channel encoded by *KCNQ1* and likewise, siRNA directed against *KCNQ1* also increased the exocytotic response. Furthermore, rs1111875 in *HHEX/IDE* was close to significantly associated with reduced exocytosis (p=0.086). Impaired exocytosis was paralleled by significant correlation to reduced number of docked granules in beta-cells from risk carriers of SNPs in or nearby *ADRA2A*, *KCNJ11*, *KCNQ1* and *HHEX/IDE*.

On the contrary, the risk genotype for rs11920090 (*SLC2A2*) associated with an increased number of docked granules. Associating the different genotypes with *in vitro* measurements of GSIS revealed that islet from donors with the *TCF7L2* rs7903146 risk genotype displayed significantly reduced GSIS, a finding also true for islets from risk carriers of *ADRA2A* rs553668.

## Genetic risk score for beta-cell dysfunction

The results of these findings were finally used in attempt to construct a genetic risk score for defective beta-cell function in the total population. For each individual, the number of risk alleles for the four SNPs (0-8) associated with secretion/exocytosis phenotypes was added. Thus, an individual homozygote for all risk alleles was assigned the score of 8. The risk score significantly predicted reduced GSIS (p=0.008) while total insulin content remained unchanged. In addition, the risk score could be associated with reduced beta-cell exocytosis (p=0.00005) which was even more pronounced in non-obese individuals (p=0.000004). The reduced exocytosis was due to both defective release of granules from the RRP and the RP without affecting the Ca<sup>2+</sup>-current. However, donors with the highest risk score had significantly reduced Ca<sup>2+</sup>-sensitivity (p=0.003) paralleled by a reduced number of docked granules (p=0.0004).

In summary:

- 1) SNPs in *TCF7L2*, *ADRA2A*, *KCNJ11*, and *KCNQ1* significantly associate with reduced exocytosis where *TCF7L2* and *ADRA2A* also correlate to reduced GSIS.
- 2) *ADRA2A*, *KCNJ11*, *KCNQ1* and *HHEX/IDE* significantly associate with reduced number of docked granules whereas *SLC2A2* associate with increased docking.
- 3) The risk score predict reduced exocytosis, GSIS, number of docked granules and Ca<sup>2+</sup>-sensitivity.

## Discussion paper IV

*KCNJ11* encodes the Kir6.2 part of the  $K_{ATP}$ -channel. However, the reduced exocytosis found in association with the rs5219 T allele cannot be ascribed to direct effects of  $K_{ATP}$  as patch-clamp circumvent the need of this channel by clamping the membrane potential artificially. Rather, the risk allele may disturb the distribution of granules in functional pools over time, a hypothesis strengthened by the finding that donors with this mutation displayed a decreased number of docked granules and reduced exocytosis of the RRP. It may be that the T-allele perturbs the opening of the channel thereby potentiating the rapid exocytosis leading to depletion of the RRP. Such a scenario is unlikely to affect the long-term refilling to the same extent. This would be reflected by a loss of the first phase insulin secretion but not of the sustained second phase secretion which is the primary contributor of insulin during 1 hour GSIS. This is in line with the finding that *KCNJ11* expression is reduced by 30% in islets from donors with T2D which could hypothetically reduce the gating of membrane potential held by these channels.

*ADRA2A* rs553668 risk allele carriers displayed a border-line significant reduction in exocytosis which could be attributed to decreased exocytosis of granules from both the RRP and the RP without changing the  $Ca^{2+}$ -current. When only non-obese donors were selected for association, both exocytosis and  $Ca^{2+}$ -sensitivity was significantly reduced. Furthermore, the SNP significantly associate with a reduced number of docked granules and GSIS, which again, showed an even stronger association in islets from lean donors. This is in line with data showing that lean patients carrying the *ADRA2A* rs553668 risk allele has a stronger correlation to T2D<sup>79</sup>. Hence, the risk allele seem to confer an effect not only in the first phase of insulin secretion but also on the second phase which is reflected by the reduced response to 1 hour GSIS. Signaling via the *ADRA2A* receptor encoded by *ADRA2A* is shown to decrease the levels of cAMP leading to the plasma membrane repolarization in mouse<sup>78</sup> thereby inhibiting exocytosis. Furthermore, a previous study by Rosengren et al., (2009) found that rs553668 associated with overexpression of the *ADRA2A* receptor in human beta-cells, directly reducing exocytosis and insulin secretion<sup>79</sup>. It is therefore tempting to speculate that rs553668 contribute to enhanced activation of the *ADRA2A* receptor thereby potentiating its negative regulation of insulin secretion. In agreement, mRNA transcripts of *ADRA2A* were not found to be reduced in islets from donors with T2D.

Total exocytosis was reduced in *TCF7L2* rs7903146 risk allele carriers whilst intracellular granule distribution was unaffected as was the depolarization-evoked  $Ca^{2+}$ -current. However, non-obese risk carriers displayed a significant reduction of the  $Ca^{2+}$ -sensitivity of exocytosis. In rodent beta-cells, reducing the levels of *TCF7L2* using RNAi result in a similar phenotypic setting of reduced exocytosis without affecting the number of docked granules<sup>176</sup>. In the human islets, the level of *TCF7L2* expression is unaltered and it is therefore tempting to speculate that rs7903146 perturbs the function of *TCF7L2*. Interestingly, silencing of *TCF7L2* was followed by increased levels of mRNA encoding *Stx1A* but decreased levels of *Stxbp1* and *ZnT8* expression in the rodent islets<sup>176</sup> which indicate that *TCF7L2* may regulate the fusion step of granules.

The risk allele of rs2237895, located in intron 15 of *KCNQ1* encoding a K<sup>+</sup>-channel, associated significantly with impaired exocytosis of the RRP in the entire cohort where again, the effect was more pronounced in islets from lean objects displaying a significant association to reduced exocytosis of granules belonging to both RRP and RP. Moreover, beta-cells from carriers of this allele exhibited a reduced number of docked granules (25% per risk allele) and also the gene expression of *KCNQ1* was not reduced in islets from donors with T2D. The finding that linopirdine as well as siRNA against *KCNQ1* rescue exocytosis in combination with the reduced number of docked granules and exocytosis found in association with the rs2237895 risk allele suggest that the SNP may potentiate the limiting effect of this channel on the burst of Ca<sup>2+</sup> action potentials. In such scenario, both priming and exocytosis of primed granules would be reduced. However, the Ca<sup>2+</sup> channel current in risk allele carriers was not significantly altered; suggesting that *KCNQ1* exerts a direct effect on exocytosis. In this study, we could not find an association with altered exocytotic response in carriers of the rs231362 SNP in *KCNQ1* previously found to correlate with T2D<sup>46</sup>, and rather this SNP associated with an increased number of docked granules.

The fact that lean individuals seem more vulnerable to genetic changes decreasing the capacity for insulin secretion may explain why these individuals develop T2D, whereas obese individuals typically display an escalating insulin release that may compensate for the reduced capacity of exocytosis. The large inter-individual differences in cellular pathways leading to reduced insulin secretion may explain why reduced exocytosis can be shown only in genetic subgroups and not in the entire T2D cohort.

The combination of genetics and functional cellular physiology used in this study has identified risk alleles associated with impaired beta-cell exocytosis and further, provided a risk score for impaired granule docking and defective Ca<sup>2+</sup>-sensitivity of exocytosis. These findings may be of future clinical use for individualized treatment strategies in subgroups of patients thus identified as benefactors of improved beta-cell exocytosis treatment.

## Paper V

### **Inhibition of rno-miR-335 enhances rapid exocytosis in insulin secreting cells through increased expression of Stxbp1**

We have previously performed a microarray analysis comparing the expression of known beta-cell specific miRNAs between the diabetic GK-rat model and the healthy Wistar rat, in which miR-335 was found to be up-regulated in the diabetic GK-rat<sup>162</sup>. Interestingly, miR-335 was shown to display potential binding sites *in silico* to mRNA 3'UTR of SNAP25 and another key player of exocytosis Stxbp1, was validated *in vitro* as a target of miR-335. We here studied the glucose-dependency of rno-miR-335, SNAP25 and Stxbp1 expression. Further we measured exocytosis and insulin secretion following rno-miR-335 inhibition.

#### **Glucose-dependent regulation of rno-miR-335, SNAP25 and Stxbp1**

The expression of miR-335, *Snap25* and *Stxbp1* in INS1-832/13 cells was studied using qRT-PCR following short-term incubation in basal and high glucose. INS1-832/13 cells were incubated for 1 hour and 2 hours in 2.8 mM or 16.7 mM glucose before mRNA/miRNA expression, protein content and insulin secretion was measured. Expression of rno-miR-335 was lower following 16.7 mM glucose stimulation as compared to 2.8 mM glucose after both 1 hour and 2 hours incubation. The reduced miRNA expression was accompanied by increased expression of *Snap25* and *Stxbp1*, where the effect on *Snap25* expression was most prominent after 1 hour whereas the *Stxbp1* expression was most prominent after 2 hour exposure to 16.7 mM glucose. GSIS was measured from the same batch of cells and insulin secretion was increased ~3-fold after incubation at 16.7 mM glucose as compared to 2.8 mM glucose.

#### **Increased expression of Stxbp1 after incubation with anti-rno-miR-335**

Next, we modulated rno-miR-335 expression by transfecting the INS1-832/13 cells with an anti-miR-335 LNA-based oligonucleotide to investigate how reduced levels of miR-335 would affect expression levels of *Snap25* and *Stxbp1*. To determine the optimal concentration of LNA-based miR-335 silencing, the cells were cultured with anti-miR-335 for 48h in different doses of the oligonucleotide. The knock-down of miR-335 was dose-dependent and maximal inhibition was observed in the highest concentration tested (50 nM). However, as visualization of the siGLO needed for the patch-clamp measurements requires higher concentrations, 160 nM LNA was used throughout the following experiments. Combining knock-down of miR-335 and 1 hour glucose stimulation revealed increased levels of *Stxbp1* in both 2.8 and 16.7 mM glucose. The relative expression of *Stxbp1* increased to the same extent at both glucose concentrations after miR-335 knock-down suggesting that glucose control the expression of *Stxbp1* through miR-335. Expression of *Snap25* remained unaltered in low glucose but was reduced following 16.7 mM glucose suggesting that *Snap25*

expression is glucose-dependent rather than directly regulated by miR-335. The increased expression of *Stxbp1* due to knock-down of miR-335 could not be demonstrated to increase glucose-stimulated insulin release (data not shown).

### **Increased exocytosis in anti-miR-335 treated INS1-832/13 cells**

*Stxbp1* is a protein known to be involved in exocytosis and thus we proceeded to study the exocytotic response in INS1-832/13 following silencing of miR-335. Standard whole-cell patch clamp capacitance measurements revealed that the membrane capacitance was ~50% higher ( $p < 0.01$ ) in cells transfected with anti-miR-335 ( $n=10$ ) compared to control ( $n=8$ ). A closer inspection of the train revealed that the increase in membrane capacitance of RRP granules was enhanced ~60% after rno-miR-335 knock-down and likewise, the increase in membrane capacitance representing exocytosis of granules refilled from the RP was also significantly higher. The increase in membrane capacitance due to miR-335 knock-down was not associated with any changes in the voltage-dependent  $Ca^{2+}$ -current.

In summary:

- 1) Short-term glucose conditioning reveal that high glucose reduces the levels of miR-335, where *Snap25* expression is most increased following 1 hour glucose stimulation whereas the effect of *Stxbp1* expression is most prominent after 2 hours.
- 2) Knock-down of miR-335 expression is paralleled by increasing expression-levels of *Stxbp1* following 1h glucose stimulation.
- 3) Inhibiting rno-miR-335 expression is associated with significantly increased exocytosis of granules belonging to both the RRP and the RP, without affecting the  $Ca^{2+}$ -current

## Discussion paper V

The expression of several genes important for exocytosis is reduced in patients with T2D<sup>22</sup> and lately, miRNAs have been suggested to be involved in the pathogenesis of T2D<sup>177</sup>. Here we investigated miR-335 and its role on insulin exocytosis.

We propose that miR-335 is a likely candidate to fine-tune the regulation of the exocytotic process since miR-335 expression decreased with increasing glucose concentration in the short time-span and associated with increased expression of *Stxbp1*. We cannot exclude that other glucose-dependent regulators are involved in decreased insulin exocytosis but the action of miR-335 on *Stxbp1* expression may well be a contributing factor in regulated exocytosis.

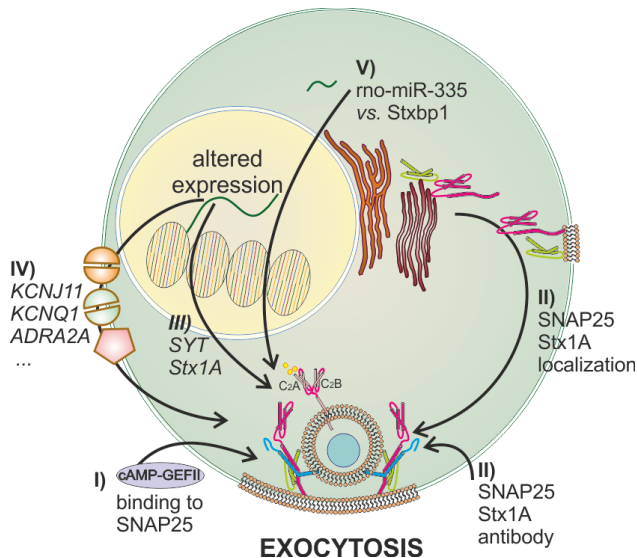
Silencing of rno-miR-335 was associated with an increased exocytotic response, but glucose stimulated insulin secretion measured over 1 hour did not change. This paradox can be explained by the dual binding partners of *Stxbp1* during the process leading to fusion of the granules<sup>178,179</sup>. Priming of docked granules has been shown to be facilitated by *Stxbp1*<sup>99</sup>. This would explain why we observed an enhanced exocytotic response after rno-miR-335 knock-down since the accompanying increased expression of *Stxbp1* promoted priming of the secretory granules. However, *Stxbp1* has also been shown to bind granuphilin, which is a known negative regulator of insulin granule exocytosis. In the case of 1 hour glucose stimulation, docking needs to be facilitated and it might be that less granuphilin disengage due to the increased level of *Stxbp1* present, thereby hindering insulin secretion. In this context it is of interest that miR-9 has been demonstrated to indirectly regulate the expression of granuphilin, and it might be that a combination of anti-miR-9 and anti-miR-335 would be an optimal cocktail to enhance both docking and priming, which should be reflected by increased insulin secretion following 1h of high glucose stimulation.

We have previously demonstrated that rno-mir-335 expression is elevated in the diabetic GK-rat islets<sup>162</sup> and in addition, the level of *Stxbp1* is down-regulated<sup>148,149</sup>. Rno-miR-335 binding to the 3'UTR of *Stxbp1* has been confirmed and in the context of this study, rno-miR-335 regulates *Stxbp1* expression. Further, *Stxbp1* has been shown to be down-regulated in beta-cells from human islets with T2D<sup>22</sup>. It would be interesting to study if miRNA is involved in *Stxbp1* regulation in which reduced expression of *Stxbp1* and decreased exocytotic response might be ameliorated by anti-miRNA treatment.

# 7 Concluding Remarks

In this thesis, aspects of exocytosis in relation to T2D have been investigated as illustrated in Fig 8. The following major conclusions were reached:

- I. The C-terminal of SNAP25 transduces cAMP-enhanced release of primed granules, whereas the N-terminal of SNAP25 is involved in PKA-dependent refilling.
- II. Localization of Stx1A and SNAP25 is glucose dependent, and these SNARE-proteins determine the temporal aspects of exocytosis without affecting the voltage-dependent  $\text{Ca}^{2+}$ -current in the alpha-cell.
- III. Expression of the exocytotic genes *Stx1A*, *SYT4*, *SYT7*, *SYT11* and *SYT13* were down-regulated in islets from human donors with T2D and correlated negatively with HbA1c and positively with GSIS.
- IV. Variants near *KCNQ1*, *KCNJ11*, *ADRA2A*, *HHEX-IDE* and *SLC2A2* affect granular docking, whereas variants near *KCNQ1*, *KCNJ11*, *ADRA2A* and *TCF7L2* associated with reduced exocytosis, which for the latter two also associated with reduced GSIS in human beta-cells.
- V. Expression of *Stxbp1* is regulated by rno-miR-335 and exocytosis increases upon knock-down of rno-miR-335 without affecting the  $\text{Ca}^{2+}$  -current.



**Fig 8** Model of factors that influence exocytosis investigated in paper I-V in this thesis.



## Perspectives

Within the scope of this thesis it is evident that exocytosis is an important part of functional glucose homeostasis and that a broad range of factors can influence the exocytotic outcome. In this context, regulation of exocytosis spans from the transcription of DNA, translation, transport and function of exocytotic proteins as well as their intricate interactions with one another. All while maintaining the correct cues in response to increased levels of intracellular  $\text{Ca}^{2+}$  either amplified by second messengers or triggered by glucose-stimulated coupling. Indeed, the multitude of events that can alter hormone secretion is far from completely depicted in this thesis and more questions remain to be asked than have been answered.

Nevertheless, several important findings have been established that could potentially aid in our goal to prevent, or find better individualized treatment of, T2D in the future. The finding that SNAP25 binds to cAMP-GEFII on the N-terminal (paper I), points towards a possibility of potentiating insulin release with cAMP-inducing agents. Further, genetic screening of patients with T2D arises as a valuable tool to better direct treatment of T2D. If so, an individual found to exhibit SNPs in ADRA2A or TCF7L2 may benefit from early introduction of beta-cell insulin-secretion inducing agents. Alternatively, a person found positive for a high risk score may earnestly pursue a lifestyle intervention if faced with increased vulnerability to develop T2D (paper IV). In this context, the emerging field of miRNA research pointing toward their being important regulators of insulin secretion also presents an opportunity of future treatment of T2D. As they are readily retrieved from blood and highly tissue-specific they can serve the double role of both acting as biomarkers of disease progression as well as future targets of treatment (paper V). More research needs to be conducted in order to establish whether the finding in paper III is a cause or consequence of T2D. Either way synaptotagmins or Stx1A could serve as possible targets of treatment either by potentiating the  $\text{Ca}^{2+}$ -induced response in exocytosis or by means of better predicting elevated future risk of developing T2D. As glucagon release is almost invariably perturbed in patients with T2D, more research on why exocytosis of glucagon is increased when blood-glucose levels are already sufficient or even elevated needs to be conducted in order to successfully counteract this action. We hope that our study (paper II), reinforcing that Stx1A and SNAP25 participate in glucagon secretion and revealing changes in localization in response to high glucose, may be of future use in this quest.

Taken together, extensive research on the molecular mechanisms participating in hormone exocytosis may well contribute to future treatment of the reduced insulin secretion apparent in T2D.

# 8 Populärvetenskaplig Sammanfattning

Typ 2 Diabetes (T2D) räknas som en folksjukdom i Sverige vilket innebär att den är så vanlig att minst 4 % av befolkningen är drabbad. Förekomsten av diabetes ökar lavinartat över hela världen och man beräknar att så många som 300 miljoner människor har diabetes idag.

## ***socker är en livsviktig energikälla, men lagom är bäst***

Socker i mat förs ut till blodet och används som energi i kroppen. För mycket socker skadar blodkärlen så att risken för hjärtinfarkt ökar, medan för låga nivåer leder till akut energibrist framförallt i hjärnan vilket kan resultera i koma. Bukspottskörteln har sensorer som känner av sockernivån i blodet och motverkar för högt eller för lågt socker genom hormonfrisättning. Vid höga sockernivåer frisätts hormonet insulin vilket signalerar att fett, musklerna, levern och hjärnan skall samla upp socker från blodet så att mängden ”fritt” socker minskar. Ifall sockernivån sjunker till för låga nivåer upphör bukspottskörteln att frisätta insulin och börjar istället leverera ett annat hormon, glukagon, som signalerar till levern att den skall frisätta socker till blodet. På så sätt balanserar bukspottskörteln, via insulin och glukagon, hela tiden blodets sockernivå.

## ***Tre källor till skadligt höga sockernivåer hos individer med T2D***

Vid T2D är tre skeden framträdande, antingen var för sig eller i kombination:

- 1) Frisättning av glukagon trots normala eller förhöjda blodsockernivåer leder till att höja sockerhalten ännu mer
- 2) Högt sockerintag leder till ökad frisättning av insulin vilket kan leda till en slags utbrändhet i fett och musklerna som ständigt stimuleras och dessa blir tillslut resistent mot insulin
- 3) Frisättningen av insulin från bukspottskörteln försämras vilket leder till att inte tillräckligt med insulin finns i omlopp för att sänka blodsockret.

I denna avhandling har jag främst undersökt det tredje skedet; Olika faktorer som kan leda till att frisättningen av insulin försämras.

## ***Hur insulin hamnar i blodet - Exocytos***

Inuti bukspottskörtelns celler ligger insulinet förpackat i kapslar. Vid höga sockernivåer smälter kapslarna samman med cellens skal så att insulinet hamnar i blodet. Denna sammansmältning kallas i vetenskapliga termer för exocytos. Exocytosen sker med hjälp av snaror på kapslarna som binder in till två kompletterande snaror (Syntaxin1A och SNAP25) i cellens skal. Vidare finns det strukturer (t.ex. Synaptotagminer) som agerar ”klistre” och förbättrar snarornas inknytning. Vår hypotes är att olika delar av exocytosen är försämrade vid T2D.

## ***T2D i vårt DNA?***

Stx1A, SNAP25 och Synaptotagmin är alla proteiner. Information som behövs för att kunna bilda proteiner finns lagrat i våra gener i spiraler av DNA-strängar inuti varje cell. Varje individs DNA genomgår spontana förändringar (mutationer). I de flesta fall gör dessa mutationer inte någon skillnad, men ibland förbättras eller försämras funktionen hos de proteiner som bildas från en muterad gen. Risken att utveckla T2D är delvis ärftlig. Därför försöker man undersöka gener för att se om det finns mutationer som är extra vanliga hos människor med diabetes, och i så fall, om mutationerna gör att motsvarande protein förändras. DNA-strängarna består inte enbart av proteinbildande gener, det finns också delar av DNA som bildar ytterst små partiklar som inte är protein. Dessa kallas för microRNA och justerar faktiskt hur mycket protein som bildas från generna och leder oftast till att mindre protein bildas.

## ***Fem studier av Exocytos***

I denna avhandling har jag i fem delar studerat olika aspekter av den exocytotiska processen:

- 1) Vi har studerat strukturen och funktionen av proteinet SNAP25 och kommit fram till att skilda delar av SNAP25 bidrar till olika steg under exocytosen av insulin.
- 2) Vi har också undersökt celler som innehåller glukagon och studerat om det finns mindre SNAP25 och Syntaxin1A i cellens skal vid förhöjda sockernivåer. Vi kom fram till att deras placering i cellen är sockerberoende och att de är nödvändiga för att exocytosen av glukagon skall fungera normalt.
- 3) Vidare har jag undersökt proteinmängder hos personer med T2D. Vi fann att individer med T2D generellt har mindre Syntaxin 1A och Synaptotagmin och att människor som har färre av dessa proteiner tenderar att ha högre blodsockernivåer, vilket vi tolkar som att de har en försämrad exocytos som bidrar till att de har mindre insulin i blodet.
- 4) Flera forskare har visat att fyra gener ofta är muterade hos människor med T2D. Vi undersökte om dessa gener parallellt ger en större effekt än om man har varje muterad gen för sig. Vi kom fram till att ju fler av dessa muterade gener en individ har, desto sämre fungerar insulinfrisättningen.
- 5) En tidigare studie har visat att råttor med diabetes har högre halter av ett specifikt microRNA som minskar mängden av ett protein som ingår i exocytosen. När vi förhindrade funktionen av detta microRNA bildades mer av proteinet och exocytosen förbättrades i insulin-innehållande celler.

## ***Sammanfattning***

Sammantaget visar våra studier att flera faktorer kan leda till en förändrad exocytos, från mutationer i gener till funktionen av proteiner, och att försämrad exocytos direkt korrelerar till försämrad insulinfrisättning. Sålunda torde dessa omständigheter bidra till förhöjda sockernivåer i T2D. Genom att noggrant utreda de exakta mekanismerna som styr den försämrade exocytosen hoppas vi att framtidens medicinering kan bli mer individualiserad och bättre riktad mot specifika proteiner eller processer för att förebygga, eller underlätta behandlingen av, skadligt förhöjda blodsockernivåer i T2D.

## 9 Acknowledgements

First and all I need to thank my supervisor, Lena Eliasson for all the help I have received during those years in the lab, structuring up my papers, this thesis, as well as my life in general. Thank you! Speaking of structure, the two backbones of the lab must of course be immensely thanked; Anna-Maria Veljanovska Ramsay and Britt-Marie Nilsson, for always lending a hand and a nice word when needed the most! And speaking of a helping hand, Jonathan Esguerra has been more of a coworker than my co-supervisor by helping me out with tons of issues ranging from statistical issues to deadline-solving superpowers: Thanks! And gratitude to my other co-supervisor Erik Renström for all things you have helped me with over the years. I also had the fortune to share office space with the kindest of postdocs Anna Wendt, Ulrika Krus and Morten Gram-Pedersen from whom I reckon I have learnt most of what I know: Thank you for always saying “yes” to my standard phrase “eh, do you have time for a question?”! I want to thank former members of Lena Eliasson’s group, Jenny Vikman in particular for sparing me endless hours making my own illustrations when I could borrow yours (really, thank you). I am also grateful for Yang De Marinis educating me in how to handle the patch-clamp microscopy (as well as Chinese history, literature and culture in general) and to Enming Zhang for teaching me the confocal laser microscopy. Thank you Inez Mollet for helping me out when I got lost in the genetic jungle not being able to figure out why my siRNA just would not work. And I wish to acknowledge my other co-authors that have contributed to this thesis, particularly Anders Olsson, Charlotte Ling and Anders Rosengren for really nice collaborations. I am so happy that Vini Nagaraj has always succeeded to cheer me up whenever we manage to meet and greet ;). And thanks to Anna Edlund, for being a great outlet of books, laughter, gossip and [scientific] discussions. On the note of scientific matters, I want to thank Thomas Reinbothe and Pawel Buda for all the nice conversations at the lab bench and in the lunch room. I am grateful to Rita Wallén for all help and patience with the EM preparations. Thanks to all other members of Lena Eliasson, Erik Renström, Albert Salehi and Anders Rosengren groups for bringing a nice atmosphere to the lab, making this PhD such a great experience!

I also wish to acknowledge funders of my PhD: Swedish Research Council, Swedish Diabetes Foundation, Knut and Alice Wallenberg Foundation, Novo Nordisk Foundation and LUDC/DPLU.

Tack, Mamma och Pappa, Per och Anette för att ni har ställt upp under alla år. Utan er kärlek, ert stöd, goda råd och ständiga uppmuntran hade jag aldrig kommit så här långt! Och tack Karin för att du tålmodigt lyssnat på mina fullständigt obegripliga förklaringar när jag ringer om något experiment, och ändå lyckats ge bra råd! Massor av kramar till Matilda och Jonathan för att ni är de bästa småsyskon jag vet! Och tusen tack till världens bästa brorsa Anders som alltid får mig att skratta även i skarpt läge, förutom möjligtvis när jag tvingas hålla i spacklen och inte du. Resten av släkten som jag inte har plats att nämna vid namn här: Tack! Thanks also to my two American Families for all love and support over the last 13 years since I first set foot in the U.S.A. I am so grateful!

Tack Anna Carlsson för att du utfodrar en hungrig doktorand då och då, och ställer upp på frågeformulär likaväl som du är en fantastisk vän (Erik är inte så dum han heller)! Jag har saknat Elin Ekström även om jag unnar dig och Alexander allt gott i NY, men det var skönt att ha en gammal sambo på labbet som kunde sticka in huvudet på kontoret och bjuda på det senaste skvallret. Tack till alla mina vänner i Lund, Malmö, Göteborg, Stockholm, Köpenhamn, Hamburg, och USA, ingen nämnd och ingen glömd!

Martin Salö, jag är så lycklig som har dig! Dessutom blir vi snart Dr Sofia och Dr Martin i lust och nöd! Tack för att du alltid stöttar mig i allt. Jag är så himla glad att din kärlek finns i mitt liv <3

# 10 References

1. Danaei, G., *et al.* National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet* **378**, 31-40 (2011).
2. Emerging Risk Factors, C., *et al.* Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet* **375**, 2215-2222 (2010).
3. Quesada, I., Tuduri, E., Ripoll, C. & Nadal, A. Physiology of the pancreatic alpha-cell and glucagon secretion: role in glucose homeostasis and diabetes. *J Endocrinol* **199**, 5-19 (2008).
4. Wierup, N., Svensson, H., Mulder, H. & Sundler, F. The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. *Regul Pept* **107**, 63-69 (2002).
5. Seaquist, E.R., Neal, A.R., Shoger, K.D., Walseth, T.F. & Robertson, R.P. G-proteins and hormonal inhibition of insulin secretion from HIT-T15 cells and isolated rat islets. *Diabetes* **41**, 1390-1399 (1992).
6. Zimmet, P., Alberti, K.G. & Shaw, J. Global and societal implications of the diabetes epidemic. *Nature* **414**, 782-787 (2001).
7. Galli, J., *et al.* Pathophysiological and genetic characterization of the major diabetes locus in GK rats. *Diabetes* **48**, 2463-2470 (1999).
8. Poulsen, P., *et al.* Heritability of insulin secretion, peripheral and hepatic insulin action, and intracellular glucose partitioning in young and old Danish twins. *Diabetes* **54**, 275-283 (2005).
9. Stumvoll, M., Goldstein, B.J. & van Haefen, T.W. Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* **365**, 1333-1346 (2005).
10. Duggirala, R., *et al.* Linkage of type 2 diabetes mellitus and of age at onset to a genetic location on chromosome 10q in Mexican Americans. *Am J Hum Genet* **64**, 1127-1140 (1999).
11. Lin, J.M., *et al.* Phenotyping of individual pancreatic islets locates genetic defects in stimulus secretion coupling to Niddm1 within the major diabetes locus in GK rats. *Diabetes* **50**, 2737-2743 (2001).
12. Hosker, J.P., Rudenski, A.S., Burnett, M.A., Matthews, D.R. & Turner, R.C. Similar reduction of first- and second-phase B-cell responses at three different glucose levels in type II diabetes and the effect of gliclazide therapy. *Metabolism* **38**, 767-772 (1989).
13. Gerich, J.E. Is reduced first-phase insulin release the earliest detectable abnormality in individuals destined to develop type 2 diabetes? *Diabetes* **51 Suppl 1**, S117-121 (2002).
14. Del Prato, S. & Tiengo, A. The importance of first-phase insulin secretion: implications for the therapy of type 2 diabetes mellitus. *Diabetes Metab Res Rev* **17**, 164-174 (2001).
15. Shah P, V.A., Basu A, Basu R, Schwenk WF, Rizza RA. Lack of suppression of glucagon contributes to postprandial hyperglycemia in subjects with type 2 diabetes mellitus. *J Clin Endocrinol Metab* **85**, 6 (2000).
16. Zobel, C., *et al.* Molecular dissection of the inward rectifier potassium current (IK1) in rabbit cardiomyocytes: evidence for heteromeric co-assembly of Kir2.1 and Kir2.2. *J Physiol* **550**, 365-372 (2003).
17. Sutton, R.B., Fasshauer, D., Jahn, R. & Brunger, A.T. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* **395**, 347-353 (1998).
18. Gauthier, B.R. & Wollheim, C.B. Synaptotagmins bind calcium to release insulin. *Am J Physiol Endocrinol Metab* **295**, E1279-1286 (2008).
19. Straub, S.G. & Sharp, G.W. Hypothesis: one rate-limiting step controls the magnitude of both phases of glucose-stimulated insulin secretion. *Am J Physiol Cell Physiol* **287**, C565-571 (2004).
20. Eliasson, L., Renstrom, E., Ding, W.G., Proks, P. & Rorsman, P. Rapid ATP-dependent priming of secretory granules precedes Ca(2+)-induced exocytosis in mouse pancreatic B-cells. *J Physiol* **503 (Pt 2)**, 399-412 (1997).
21. Eliasson, L., *et al.* Novel aspects of the molecular mechanisms controlling insulin secretion. *J Physiol* **586**, 3313-3324 (2008).
22. Ostenson, C.G., Gaisano, H., Sheu, L., Tibell, A. & Bartfai, T. Impaired gene and protein expression of exocytotic soluble N-ethylmaleimide attachment protein receptor complex proteins in pancreatic islets of type 2 diabetic patients. *Diabetes* **55**, 435-440 (2006).

23. Owerbach, D., Bell, G.I., Rutter, W.J. & Shows, T.B. The insulin gene is located on chromosome 11 in humans. *Nature* **286**, 82-84 (1980).
24. Berne, R.M., Levy, M.N., Koeppen, B.M. & Stanton, B.A. *Physiology*, (Mosby ;, St. Louis, Mo. London,, 2004).
25. Bell, G.I., Sanchez-Pescador, R., Laybourn, P.J. & Najarian, R.C. Exon duplication and divergence in the human preproglucagon gene. *Nature* **304**, 368-371 (1983).
26. Orskov, C., Holst, J.J., Poulsen, S.S. & Kirkegaard, P. Pancreatic and intestinal processing of proglucagon in man. *Diabetologia* **30**, 874-881 (1987).
27. Novak, U., Wilks, A., Buell, G. & McEwen, S. Identical mRNA for preproglucagon in pancreas and gut. *Eur J Biochem* **164**, 553-558 (1987).
28. Mojsov, S., *et al.* Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J Biol Chem* **261**, 11880-11889 (1986).
29. George, S.K., Uttenthal, L.O., Ghiglione, M. & Bloom, S.R. Molecular forms of glucagon-like peptides in man. *FEBS Lett* **192**, 275-278 (1985).
30. Bataille, D. Pro-protein convertases in intermediary metabolism: islet hormones, brain/gut hormones and integrated physiology. *Journal of molecular medicine* **85**, 673-684 (2007).
31. Orskov, C., Bersani, M., Johnsen, A.H., Hojrup, P. & Holst, J.J. Complete sequences of glucagon-like peptide-1 from human and pig small intestine. *J Biol Chem* **264**, 12826-12829 (1989).
32. Meglasson, M.D. & Matschinsky, F.M. Pancreatic islet glucose metabolism and regulation of insulin secretion. *Diabetes/metabolism reviews* **2**, 163-214 (1986).
33. Henquin, J.C. & Meissner, H.P. Significance of ionic fluxes and changes in membrane potential for stimulus-secretion coupling in pancreatic B-cells. *Experientia* **40**, 1043-1052 (1984).
34. Ammala, C., *et al.* Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. *J Physiol* **472**, 665-688 (1993).
35. Aittoniemi, J., *et al.* Review. SUR1: a unique ATP-binding cassette protein that functions as an ion channel regulator. *Philos Trans R Soc Lond B Biol Sci* **364**, 257-267 (2009).
36. Ashcroft, F.M. & Gribble, F.M. ATP-sensitive K<sup>+</sup> channels and insulin secretion: their role in health and disease. *Diabetologia* **42**, 903-919 (1999).
37. Ashcroft, F.M. & Rorsman, P. Electrophysiology of the pancreatic beta-cell. *Prog Biophys Mol Biol* **54**, 87-143 (1989).
38. Rorsman, P., *et al.* The Cell Physiology of Biphasic Insulin Secretion. *News Physiol Sci* **15**, 72-77 (2000).
39. Wollheim, C.B. & Sharp, G.W. Regulation of insulin release by calcium. *Physiol Rev* **61**, 914-973 (1981).
40. MacDonald, P.E. Signal integration at the level of ion channel and exocytotic function in pancreatic beta-cells. *Am J Physiol Endocrinol Metab* **301**, E1065-1069 (2011).
41. Henquin, J.C. Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **49**, 1751-1760 (2000).
42. Braun, M., *et al.* Voltage-gated ion channels in human pancreatic {beta}-cells Electrophysiological characterization and role in insulin secretion. *Diabetes* (2008).
43. Yang, S.N. & Berggren, P.O. The role of voltage-gated calcium channels in pancreatic beta-cell physiology and pathophysiology. *Endocr Rev* **27**, 621-676 (2006).
44. Ashcroft, F.M. & Rorsman, P. Diabetes Mellitus and the beta Cell: The Last Ten Years. *Cell* **148**, 1160-1171 (2012).
45. Maechler, P. & Wollheim, C.B. Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* **402**, 685-689 (1999).
46. Straub, S.G. & Sharp, G.W. Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes Metab Res Rev* **18**, 451-463 (2002).
47. Ammon, H.P. & Steinke, J. 6-Ammonicotinamide (6-AN) as a diabetogenic agent. In vitro and in vivo studies in the rat. *Diabetes* **21**, 143-148 (1972).
48. Detimary, P., *et al.* The changes in adenine nucleotides measured in glucose-stimulated rodent islets occur in beta cells but not in alpha cells and are also observed in human islets. *J Biol Chem* **273**, 33905-33908 (1998).
49. Gopel, S.O., *et al.* Regulation of glucagon release in mouse -cells by KATP channels and inactivation of TTX-sensitive Na<sup>+</sup> channels. *J Physiol* **528**, 509-520 (2000).
50. Macdonald, P.E., *et al.* A KATP Channel-Dependent Pathway within alpha Cells Regulates Glucagon Release from Both Rodent and Human Islets of Langerhans. *PLoS Biol* **5**, e143 (2007).
51. Gopel, S., Kanno, T., Barg, S., Galvanovskis, J. & Rorsman, P. Voltage-gated and resting membrane currents recorded from B-cells in intact mouse pancreatic islets. *J Physiol* **521 Pt 3**, 717-728 (1999).
52. Gromada, J., *et al.* Adrenaline stimulates glucagon secretion in pancreatic A-cells by increasing the Ca<sub>2</sub><sup>+</sup> current and the number of granules close to the L-type Ca<sub>2</sub><sup>+</sup> channels. *J Gen Physiol* **110**, 217-228 (1997).

53. Barg, S., Galvanovskis, J., Gopel, S.O., Rorsman, P. & Eliasson, L. Tight coupling between electrical activity and exocytosis in mouse glucagon-secreting alpha-cells. *Diabetes* **49**, 1500-1510 (2000).
54. Ishihara, H., Maechler, P., Gjinovci, A., Herrera, P.L. & Wollheim, C.B. Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells. *Nat Cell Biol* **5**, 330-335 (2003).
55. Le Marchand, S.J. & Piston, D.W. Glucose suppression of glucagon secretion: metabolic and calcium responses from alpha-cells in intact mouse pancreatic islets. *J Biol Chem* **285**, 14389-14398 (2010).
56. Vieira, E., Salehi, A. & Gylfe, E. Glucose inhibits glucagon secretion by a direct effect on mouse pancreatic alpha cells. *Diabetologia* **50**, 370-379 (2007).
57. Dean, P.M. Ultrastructural morphometry of the pancreatic -cell. *Diabetologia* **9**, 115-119 (1973).
58. Olofsson, C.S., *et al.* Fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic B-cells. *Pflugers Arch* **444**, 43-51 (2002).
59. Rorsman, P. & Renstrom, E. Insulin granule dynamics in pancreatic beta cells. *Diabetologia* **46**, 1029-1045 (2003).
60. Gopel, S., *et al.* Capacitance measurements of exocytosis in mouse pancreatic alpha-, beta- and delta-cells within intact islets of Langerhans. *J Physiol* **556**, 711-726 (2004).
61. Ostlund, R.E., Jr. Contractile proteins and pancreatic beta-cell secretion. *Diabetes* **26**, 245-252 (1977).
62. Meng, Y.X., Wilson, G.W., Avery, M.C., Varden, C.H. & Balczon, R. Suppression of the expression of a pancreatic beta-cell form of the kinesin heavy chain by antisense oligonucleotides inhibits insulin secretion from primary cultures of mouse beta-cells. *Endocrinology* **138**, 1979-1987 (1997).
63. Orci, L., Gabbay, K.H. & Malaisse, W.J. Pancreatic beta-cell web: its possible role in insulin secretion. *Science* **175**, 1128-1130 (1972).
64. Ivarsson, R., Jing, X., Waselle, L., Regazzi, R. & Renstrom, E. Myosin 5a controls insulin granule recruitment during late-phase secretion. *Traffic* **6**, 1027-1035 (2005).
65. Barg, S., Eliasson, L., Renstrom, E. & Rorsman, P. A subset of 50 secretory granules in close contact with L-type Ca<sup>2+</sup> channels accounts for first-phase insulin secretion in mouse beta-cells. *Diabetes* **51 Suppl 1**, S74-S82 (2002).
66. Wisner, O., *et al.* The voltage sensitive Lc-type Ca<sup>2+</sup> channel is functionally coupled to the exocytotic machinery. *Proc Natl Acad Sci U S A* **96**, 248-253 (1999).
67. Barg, S., *et al.* Fast exocytosis with few Ca(2+) channels in insulin-secreting mouse pancreatic B cells. *Biophysical journal* **81**, 3308-3323 (2001).
68. Jing, X., *et al.* CaV2.3 calcium channels control second-phase insulin release. *J Clin Invest* **115**, 146-154 (2005).
69. Lemmens, R., Larsson, O., Berggren, P.O. & Islam, M.S. Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release from the endoplasmic reticulum amplifies the Ca<sup>2+</sup> signal mediated by activation of voltage-gated L-type Ca<sup>2+</sup> channels in pancreatic beta-cells. *J Biol Chem* **276**, 9971-9977 (2001).
70. Hao, M., *et al.* Regulation of two insulin granule populations within the reserve pool by distinct calcium sources. *J Cell Sci* **118**, 5873-5884 (2005).
71. Alberts, B., Wilson, J.H. & Hunt, T. *Molecular biology of the cell*, (Garland Science, New York, 2008).
72. Ammala, C., Ashcroft, F.M. & Rorsman, P. Calcium-independent potentiation of insulin release by cyclic AMP in single beta-cells. *Nature* **363**, 356-358 (1993).
73. Renstrom, E., Eliasson, L. & Rorsman, P. Protein kinase A-dependent and -independent stimulation of exocytosis by cAMP in mouse pancreatic B-cells. *J Physiol* **502 ( Pt 1)**, 105-118 (1997).
74. Ozaki, N., *et al.* cAMP-GEFII is a direct target of cAMP in regulated exocytosis. *Nat Cell Biol* **2**, 805-811 (2000).
75. Vikman, J., *et al.* Truncation of SNAP-25 reduces the stimulatory action of cAMP on rapid exocytosis in insulin-secreting cells. *Am J Physiol Endocrinol Metab* **297**, E452-461 (2009).
76. Eliasson, L., *et al.* SUR1 regulates insulin-independent cAMP-induced granule priming in mouse pancreatic B-cells. *J Gen Physiol* **121**, 181-197 (2003).
77. Ullrich, S., Prentki, M. & Wollheim, C.B. Somatostatin inhibition of Ca<sup>2+</sup>(+)-induced insulin secretion in permeabilized HIT-T15 cells. *Biochem J* **270**, 273-276 (1990).
78. Peterhoff, M., *et al.* Inhibition of insulin secretion via distinct signaling pathways in alpha2-adrenoceptor knockout mice. *Eur J Endocrinol* **149**, 343-350 (2003).
79. Rosengren, A.H., *et al.* Overexpression of alpha2A-adrenergic receptors contributes to type 2 diabetes. *Science* **327**, 217-220 (2010).
80. De Marinis, Y.Z., *et al.* GLP-1 inhibits and adrenaline stimulates glucagon release by differential modulation of N- and L-type Ca<sup>2+</sup> channel-dependent exocytosis. *Cell Metab* **11**, 543-553 (2010).
81. Sudhof, T.C. & Rizo, J. Synaptic vesicle exocytosis. *Cold Spring Harb Perspect Biol* **3**(2011).
82. Leung, Y.M., Kwan, E.P., Ng, B., Kang, Y. & Gaisano, H.Y. SNAREing voltage-gated K<sup>+</sup> and ATP-sensitive K<sup>+</sup> channels: tuning beta-cell excitability with syntaxin-1A and other exocytotic proteins. *Endocr Rev* **28**, 653-663 (2007).

83. Hou, J.C., Min, L. & Pessin, J.E. Insulin granule biogenesis, trafficking and exocytosis. *Vitam Horm* **80**, 473-506 (2009).
84. Ohara-Imaizumi, M., *et al.* Correlation of syntaxin-1 and SNAP-25 clusters with docking and fusion of insulin granules analysed by total internal reflection fluorescence microscopy. *Diabetologia* **47**, 2200-2207 (2004).
85. Vikman, J., Ma, X., Hockerman, G.H., Rorsman, P. & Eliasson, L. Antibody inhibition of synaptosomal protein of 25 kDa (SNAP-25) and syntaxin 1 reduces rapid exocytosis in insulin-secreting cells. *J Mol Endocrinol.* **36**, 503-515 (2006).
86. Zhang, W., *et al.* Down-regulated expression of exocytotic proteins in pancreatic islets of diabetic GK rats. *Biochem Biophys Res Commun* **291**, 1038-1044 (2002).
87. Barg, S., Knowles, M.K., Chen, X., Midorikawa, M. & Almers, W. Syntaxin clusters assemble reversibly at sites of secretory granules in live cells. *Proc Natl Acad Sci U S A* **107**, 20804-20809 (2010).
88. Salaun, C., James, D.J., Greaves, J. & Chamberlain, L.H. Plasma membrane targeting of exocytic SNARE proteins. *Biochim Biophys Acta* **1693**, 81-89 (2004).
89. Vikman, J., Jimenez-Feltstrom, J., Nyman, P., Thelin, J. & Eliasson, L. Insulin secretion is highly sensitive to desorption of plasma membrane cholesterol. *FASEB J* **23**, 58-67 (2009).
90. Wang, Z. & Thurmond, D.C. Mechanisms of biphasic insulin-granule exocytosis - roles of the cytoskeleton, small GTPases and SNARE proteins. *J Cell Sci* **122**, 893-903 (2009).
91. Ohara-Imaizumi, M., *et al.* Imaging analysis reveals mechanistic differences between first- and second-phase insulin exocytosis. *J Cell Biol* **177**, 695-705 (2007).
92. Lam, P.P., *et al.* Transgenic mouse overexpressing syntaxin-1A as a diabetes model. *Diabetes* **54**, 2744-2754 (2005).
93. Jeans, A.F., *et al.* A dominant mutation in Snap25 causes impaired vesicle trafficking, sensorimotor gating, and ataxia in the blind-drunk mouse. *Proc Natl Acad Sci U S A* **104**, 2431-2436 (2007).
94. Gerber, S.H. & Sudhof, T.C. Molecular determinants of regulated exocytosis. *Diabetes* **51 Suppl 1**, S3-11 (2002).
95. Mandic, S.A., *et al.* Munc18-1 and Munc18-2 proteins modulate beta-cell Ca<sup>2+</sup> sensitivity and kinetics of insulin exocytosis differently. *J Biol Chem* **286**, 28026-28040 (2011).
96. Han, G.A., *et al.* Munc18-1 domain-1 controls vesicle docking and secretion by interacting with syntaxin-1 and chaperoning it to the plasma membrane. *Molecular biology of the cell* **22**, 4134-4149 (2011).
97. Dong, Y., Wan, Q., Yang, X., Bai, L. & Xu, P. Interaction of Munc18 and Syntaxin in the regulation of insulin secretion. *Biochemical and biophysical research communications* **360**, 609-614 (2007).
98. Zhang, W., *et al.* Munc-18 associates with syntaxin and serves as a negative regulator of exocytosis in the pancreatic beta -cell. *J Biol Chem* **275**, 41521-41527 (2000).
99. Tomas, A., Meda, P., Regazzi, R., Pessin, J.E. & Halban, P.A. Munc 18-1 and granuphilin collaborate during insulin granule exocytosis. *Traffic* **9**, 813-832 (2008).
100. Lilja, L., *et al.* Cyclin-dependent kinase 5 associated with p39 promotes Munc18-1 phosphorylation and Ca(2+)-dependent exocytosis. *J Biol Chem* **279**, 29534-29541 (2004).
101. Rickman, C., Medine, C.N., Bergmann, A. & Duncan, R.R. Functionally and spatially distinct modes of munc18-syntaxin 1 interaction. *J Biol Chem* **282**, 12097-12103 (2007).
102. Dulubova, I., *et al.* Munc18-1 binds directly to the neuronal SNARE complex. *Proc Natl Acad Sci U S A* **104**, 2697-2702 (2007).
103. Toonen, R.F. & Verhage, M. Munc18-1 in secretion: lonely Munc joins SNARE team and takes control. *Trends Neurosci* **30**, 564-572 (2007).
104. Shen, J., Taresté, D.C., Paumet, F., Rothman, J.E. & Melia, T.J. Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell* **128**, 183-195 (2007).
105. McNew, J.A. Regulation of SNARE-mediated membrane fusion during exocytosis. *Chemical reviews* **108**, 1669-1686 (2008).
106. Iezzi, M., Regazzi, R. & Wollheim, C.B. The Rab3-interacting molecule RIM is expressed in pancreatic beta-cells and is implicated in insulin exocytosis. *FEBS Lett* **474**, 66-70 (2000).
107. Martinez, O. & Goud, B. Rab proteins. *Biochim Biophys Acta* **1404**, 101-112 (1998).
108. Lupashin, V.V. & Waters, M.G. t-SNARE activation through transient interaction with a rab-like guanosine triphosphatase. *Science* **276**, 1255-1258 (1997).
109. Coppola, T., *et al.* Direct interaction of the Rab3 effector RIM with Ca<sup>2+</sup> channels, SNAP-25, and synaptotagmin. *J Biol Chem* **276**, 32756-32762 (2001).
110. Gandini, M.A., *et al.* Functional Coupling of Rab3-interacting Molecule 1 (RIM1) and L-type Ca<sup>2+</sup> Channels in Insulin Release. *J Biol Chem* **286**, 15757-15765 (2011).
111. Yasuda, T., *et al.* Rim2alpha determines docking and priming states in insulin granule exocytosis. *Cell Metab* **12**, 117-129 (2010).



112. Marqueze, B., Berton, F. & Seagar, M. Synaptotagmins in membrane traffic: which vesicles do the tagmins tag? *Biochimie* **82**, 409-420 (2000).
113. McMahon, H.T. & Sudhof, T.C. Synaptic core complex of synaptobrevin, syntaxin, and SNAP25 forms high affinity alpha-SNAP binding site. *J Biol Chem* **270**, 2213-2217 (1995).
114. Ubach, J., Zhang, X., Shao, X., Sudhof, T.C. & Rizo, J. Ca<sup>2+</sup> binding to synaptotagmin: how many Ca<sup>2+</sup> ions bind to the tip of a C2-domain? *Embo J* **17**, 3921-3930 (1998).
115. Brose, N., Petrenko, A.G., Sudhof, T.C. & Jahn, R. Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science* **256**, 1021-1025 (1992).
116. Fukuda, M. & Mikoshiba, K. Characterization of KIAA1427 protein as an atypical synaptotagmin (Syt XIII). *Biochem J* **354**, 249-257 (2001).
117. Grise, F., Taib, N., Monterrat, C., Lagree, V. & Lang, J. Distinct roles of the C2A and the C2B domain of the vesicular Ca<sup>2+</sup> sensor synaptotagmin 9 in endocrine beta-cells. *Biochem J* **403**, 483-492 (2007).
118. Parton, L.E., *et al.* Limited role for SREBP-1c in defective glucose-induced insulin secretion from Zucker diabetic fatty rat islets: a functional and gene profiling analysis. *Am J Physiol Endocrinol Metab* **291**, E982-994 (2006).
119. Gao, Z., Reavey-Cantwell, J., Young, R.A., Jegier, P. & Wolf, B.A. Synaptotagmin III/VII isoforms mediate Ca<sup>2+</sup>-induced insulin secretion in pancreatic islet beta -cells. *J Biol Chem* **275**, 36079-36085 (2000).
120. Gut, A., *et al.* Expression and localisation of synaptotagmin isoforms in endocrine beta-cells: their function in insulin exocytosis. *J Cell Sci* **114**, 1709-1716 (2001).
121. Adolfsen, B. & Littleton, J.T. Genetic and molecular analysis of the synaptotagmin family. *Cell Mol Life Sci* **58**, 393-402 (2001).
122. Davis, A.F., *et al.* Kinetics of synaptotagmin responses to Ca<sup>2+</sup> and assembly with the core SNARE complex onto membranes. *Neuron* **24**, 363-376 (1999).
123. Schiavo, G., Stenbeck, G., Rothman, J.E. & Sollner, T.H. Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. *Proc Natl Acad Sci U S A* **94**, 997-1001 (1997).
124. Gerona, R.R., Larsen, E.C., Kowalchuk, J.A. & Martin, T.F. The C terminus of SNAP25 is essential for Ca(2+)-dependent binding of synaptotagmin to SNARE complexes. *J Biol Chem* **275**, 6328-6336 (2000).
125. Chapman, E.R. How does synaptotagmin trigger neurotransmitter release? *Annual review of biochemistry* **77**, 615-641 (2008).
126. Lynch, K.L., *et al.* Synaptotagmin C2A loop 2 mediates Ca<sup>2+</sup>-dependent SNARE interactions essential for Ca<sup>2+</sup>-triggered vesicle exocytosis. *Mol Biol Cell* **18**, 4957-4968 (2007).
127. von Poser, C., Ichtchenko, K., Shao, X., Rizo, J. & Sudhof, T.C. The evolutionary pressure to inactivate. A subclass of synaptotagmins with an amino acid substitution that abolishes Ca<sup>2+</sup> binding. *J Biol Chem* **272**, 14314-14319 (1997).
128. Fukuda, M., Kojima, T. & Mikoshiba, K. Phospholipid composition dependence of Ca<sup>2+</sup>-dependent phospholipid binding to the C2A domain of synaptotagmin IV. *J Biol Chem* **271**, 8430-8434 (1996).
129. Machado, H.B., Liu, W., Vician, L.J. & Herschman, H.R. Synaptotagmin IV overexpression inhibits depolarization-induced exocytosis in PC12 cells. *J Neurosci Res* **76**, 334-341 (2004).
130. Wang, C.T., *et al.* Synaptotagmin modulation of fusion pore kinetics in regulated exocytosis of dense-core vesicles. *Science* **294**, 1111-1115 (2001).
131. Thomas, D.M., Ferguson, G.D., Herschman, H.R. & Elferink, L.A. Functional and biochemical analysis of the C2 domains of synaptotagmin IV. *Mol Biol Cell* **10**, 2285-2295 (1999).
132. Gustavsson, N., *et al.* Impaired insulin secretion and glucose intolerance in synaptotagmin-7 null mutant mice. *Proc Natl Acad Sci U S A* **105**, 3992-3997 (2008).
133. Li, C., *et al.* Ca(2+)-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature* **375**, 594-599 (1995).
134. Gustavsson, N., *et al.* Synaptotagmin-7 is a principal Ca<sup>2+</sup> sensor for Ca<sup>2+</sup> -induced glucagon exocytosis in pancreas. *J Physiol* **587**, 1169-1178 (2009).
135. von Poser, C. & Sudhof, T.C. Synaptotagmin 13: structure and expression of a novel synaptotagmin. *European journal of cell biology* **80**, 41-47 (2001).
136. Venter, J.C., *et al.* The sequence of the human genome. *Science* **291**, 1304-1351 (2001).
137. Lander, E.S., *et al.* Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921 (2001).
138. International Human Genome Sequencing, C. Finishing the euchromatic sequence of the human genome. *Nature* **431**, 931-945 (2004).
139. Reich, D.E., Gabriel, S.B. & Altshuler, D. Quality and completeness of SNP databases. *Nat Genet* **33**, 457-458 (2003).
140. Kruglyak, L. & Nickerson, D.A. Variation is the spice of life. *Nat Genet* **27**, 234-236 (2001).

141. International HapMap, C. The International HapMap Project. *Nature* **426**, 789-796 (2003).
142. Saxena, R., *et al.* Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* **316**, 1331-1336 (2007).
143. Koeck, T., *et al.* A common variant in TFB1M is associated with reduced insulin secretion and increased future risk of type 2 diabetes. *Cell Metab* **13**, 80-91 (2011).
144. Lysenko, V., *et al.* Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes. *J Clin Invest* **117**, 2155-2163 (2007).
145. Olsson, A.H., *et al.* Two common genetic variants near nuclear-encoded OXPHOS genes are associated with insulin secretion in vivo. *Eur J Endocrinol* **164**, 765-771 (2011).
146. Voight, B.F., *et al.* Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat Genet* **42**, 579-589 (2010).
147. Ullrich, S., *et al.* Effects of I(Ks) channel inhibitors in insulin-secreting INS-1 cells. *Pflugers Arch* **451**, 428-436 (2005).
148. Zhang, W., *et al.* Down-regulated expression of exocytotic proteins in pancreatic islets of diabetic GK rats. *Biochemical and biophysical research communications* **291**, 1038-1044 (2002).
149. Gaisano, H.Y., Ostenson, C.G., Sheu, L., Wheeler, M.B. & Efendic, S. Abnormal expression of pancreatic islet exocytotic soluble N-ethylmaleimide-sensitive factor attachment protein receptors in Goto-Kakizaki rats is partially restored by phlorizin treatment and accentuated by high glucose treatment. *Endocrinology* **143**, 4218-4226 (2002).
150. Sonestedt, E., *et al.* Genetic Variation in the Glucose-Dependent Insulinotropic Polypeptide Receptor Modifies the Association between Carbohydrate and Fat Intake and Risk of Type 2 Diabetes in the Malmo Diet and Cancer Cohort. *J Clin Endocrinol Metab* (2012).
151. Ling, C. & Groop, L. Epigenetics: a molecular link between environmental factors and type 2 diabetes. *Diabetes* **58**, 2718-2725 (2009).
152. Bartel, D.P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215-233 (2009).
153. He, L. & Hannon, G.J. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* **5**, 522-531 (2004).
154. Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-297 (2004).
155. Ramkissoon, S.H., *et al.* Hematopoietic-specific microRNA expression in human cells. *Leukemia research* **30**, 643-647 (2006).
156. Lovis, P., Gattesco, S. & Regazzi, R. Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. *Biological chemistry* **389**, 305-312 (2008).
157. Plaisance, V., *et al.* MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. *J Biol Chem* **281**, 26932-26942 (2006).
158. Poy, M.N., *et al.* A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* **432**, 226-230 (2004).
159. Zhao, H., *et al.* Up-regulated pancreatic tissue microRNA-375 associates with human type 2 diabetes through beta-cell deficit and islet amyloid deposition. *Pancreas* **39**, 843-846 (2010).
160. Bolmeson, C., *et al.* Differences in islet-enriched miRNAs in healthy and glucose intolerant human subjects. *Biochem Biophys Res Commun* **404**, 16-22 (2011).
161. Roggli, E., *et al.* Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells. *Diabetes* **59**, 978-986 (2010).
162. Esguerra, J.L., Bolmeson, C., Cilio, C.M. & Eliasson, L. Differential glucose-regulation of microRNAs in pancreatic islets of non-obese type 2 diabetes model Goto-Kakizaki rat. *PLoS ONE* **6**, e18613 (2011).
163. *Practical methods in electron microscopy*, (Elsevier, 335 Jan Van Galenstraat, Amsterdam, Holland, 1980).
164. Hamill, O.P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F.J. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* **391**, 85-100 (1981).
165. Grant, S.F., *et al.* Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. *Nat Genet* **38**, 320-323 (2006).
166. Pinheiro, J.C. & Bates, D.M. *Mixed-effects models in S and S-PLUS*, (Springer Verlag, 2009).
167. Nagy, G., *et al.* Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. *Neuron* **41**, 417-429 (2004).
168. De Marinis, Y.Z., *et al.* Enhancement of glucagon secretion in mouse and human pancreatic alpha cells by protein kinase C (PKC) involves intracellular trafficking of PKCalpha and PKCdelta. *Diabetologia* **53**, 717-729 (2010).
169. Fujita, Y., *et al.* Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C: its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. *J Biol Chem* **271**, 7265-7268 (1996).

170. Romeo, S., *et al.* Search for genetic variants of the SYNTAXIN 1A (STX1A) gene: the -352 A>T variant in the STX1A promoter associates with impaired glucose metabolism in an Italian obese population. *International journal of obesity* **32**, 413-420 (2008).
171. Tsunoda, K., Sanke, T., Nakagawa, T., Furuta, H. & Nanjo, K. Single nucleotide polymorphism (D68D, T to C) in the syntaxin 1A gene correlates to age at onset and insulin requirement in Type II diabetic patients. *Diabetologia* **44**, 2092-2097 (2001).
172. Kaneko, K., *et al.* Class IA phosphatidylinositol 3-kinase in pancreatic beta cells controls insulin secretion by multiple mechanisms. *Cell Metab* **12**, 619-632 (2010).
173. Gauthier, B.R. & Wollheim, C.B. MicroRNAs: 'ribo-regulators' of glucose homeostasis. *Nat Med* **12**, 36-38 (2006).
174. Sladek, R., *et al.* A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* **445**, 881-885 (2007).
175. Zeggini, E., *et al.* Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nat Genet* **40**, 638-645 (2008).
176. da Silva Xavier, G., *et al.* TCF7L2 regulates late events in insulin secretion from pancreatic islet  $\beta$ -cells. in *Diabetes*, Vol. 00 0000-0000 (2009).
177. Guay, C., Roggli, E., Nesca, V., Jacovetti, C. & Regazzi, R. Diabetes mellitus, a microRNA-related disease? *Transl Res* **157**, 253-264 (2011).
178. Shi, L., Kummel, D., Coleman, J., Melia, T.J. & Giraud, C.G. Dual roles of Munc18-1 rely on distinct binding modes of the central cavity with Stx1A and SNARE complex. *Molecular biology of the cell* **22**, 4150-4160 (2011).
179. Gulyas-Kovacs, A., *et al.* Munc18-1: sequential interactions with the fusion machinery stimulate vesicle docking and priming. *J Neurosci* **27**, 8676-8686 (2007).