Modern roles for an ancient system. Intracellular Complement in the regulation of β cell function

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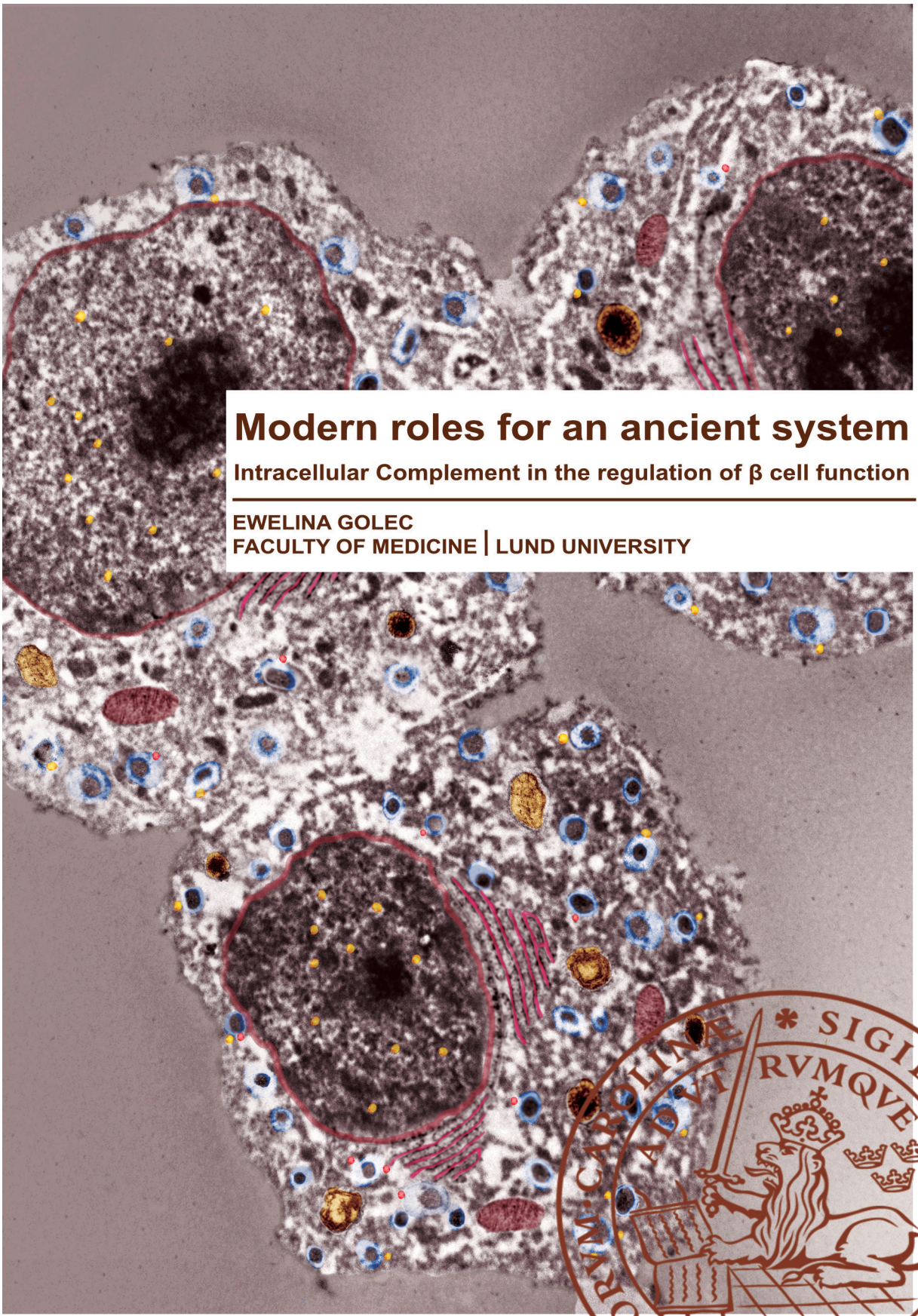

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Intracellular Complement in the regulation of β cell function

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Ewelina Golec

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract:

Type 2 diabetes (T2D) is characterized by defective insulin exocytosis from the pancreatic β-cells, accompanied by insulin resistance. Reduced β-cells mass is often seen in T2D individuals, caused by enhanced β-cells apoptosis. It is now understood that several components drive β-cells dysfunction and apoptosis. These are glucose- and lipotoxicity, ER stress, pro-inflammatory cytokines, dysfunctions in autophagy, and β-cells dedifferentiation.

Our studies revealed novel functions of intracellular complement components in β-cells, presenting a new link between complement and diabetes development.

We found that C3 is upregulated in pancreatic islets during T2D as a factor against β-cells dysfunction caused by attenuated autophagy. In paper I, we revealed a high expression of C3 in human pancreatic islets. C3 was found intracellularly in isolated human pancreatic β-cells. We verified the binding between C3 and ATG16L1 within the cytosol. C3 was required to maintain autophagy activity in β-cells, as evidenced by the massive accumulation of LC3-II puncta, indicating that in the absence of C3 autophagosomes do not fuse with lysosomes. Autophagy protects the β-cells from injuries caused by exposure to stressors, such as lipotoxicity. When exposing the C3-knockout INS-1 cells to β-cells autophagy inducers (palmitate and IAPP), we observed significantly increased cell death caused by autophagy insufficiency.

In paper II, we showed that silencing of CD59 expression in rat β-cells significantly suppressed insulin secretion. Moreover, removing the membrane-bound CD59 did not affect insulin secretion, suggesting that intracellular CD59 is involved in this function. We found that the CD59 mutant, lacking the GPI-anchor, was present intracellularly in the β-cell line. Non-GPI anchored CD59 interacts with SNARE protein: VAMP2 and rescues insulin secretion. We showed that the GPI-anchor, which is necessary for CD59 complement inhibitory function, is not necessary for its ability to mediate insulin secretion. Two other mutations: W40E and C64Y, rescued insulin secretion. Studies showed that these mutations result in a loss of CD59 complement inhibitory functions. Our data suggest that there are different structural requirements for separate functions of CD59, which are: MAC inhibition and insulin secretion.

In papers III and IV, using RNA sequencing, we revealed the presence of two CD59 isoforms lacking the GPI anchoring domain (replaced with the unique C-terminal domains). We named these isoforms IRIS-1 and IRIS-2 (Isoforms Rescuing Insulin Secretion 1 and 2). Both isoforms exist in human and mouse pancreatic islets. They colocalize with insulin granules and interact with SNARE exocytotic machinery, allowing for insulin secretion. Induction of glucotoxicity in primary, healthy human islets led to a significant decrease in IRIS-1 protein-level expression. We found that expression of both IRIS-1 and IRIS-2 is markedly reduced in islets isolated from T2D patients compared to healthy controls, suggesting that hyperglycemia may be one of the factors resulting in reduced IRIS-1 and IRIS-2 expression in T2D individuals. Next, an electropositive patch was found in the C-terminal region of IRIS-1, suggesting potential interaction with DNA. We found that IRIS-1 localizes in the nuclei of pancreatic β-cells. We confirmed that the C-terminal domain of IRIS-1 is localizing it to the nucleus. Since robust localization of IRIS-1 in the nucleus is observed only in some nuclei, it can suggest that IRIS-1 is localizing in the nucleus depending on the differentiation state of the cells or in a subset of cells with different functional relevance. We found that IRIS-1 expressing cells displayed significantly higher expression levels of *Urocortin3* and *Pdx1* (markers of mature β-cells, which loss marks the beginning of β-cells dedifferentiation), suggesting that IRIS-1 may be required for maintaining β-cells identity and function.

Key words:

Complement system, diabetes mellitus, insulin secretion, CD59, IRIS-1, IRIS-2, C3, SNARE proteins.

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The cover image represents an electron microscopy photo of rat β cells (INS-1 832/13) with a knockout of the CD59 gene. Presented cells overexpress human CD59 isoforms IRIS-1 and IRIS-2. Both isoforms were immunogold-labeled. IRIS-1 (yellow), IRIS-2 (red), insulin granules (light blue). The image has been captured and modified for artistic purposes by Ewelina Golec.

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MADE IN SWEDEN

“What I love about science is that as you learn, you don’t really get answers. You just get better questions”

John Green

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I. King BC*, Kulak K*, Krus U, Rosberg R, **Golec E**, Wozniak K, Gomez MF, Zhang E, O'Connell DJ, Renström E, Blom AM. Complement Component C3 Is Highly Expressed in Human Pancreatic Islets and Prevents β Cell Death via ATG16L1 Interaction and Autophagy Regulation. *Cell Metabolism. 2019; 29(1):202-210. Doi: 10.1016/ j.cmet.2018.09.009.*
* equal contribution

II. **Golec E**, Rosberg R, Zhang E, Renström E, Blom AM*, King BC* A cryptic non-GPI-anchored cytosolic isoform of CD59 controls insulin exocytosis in pancreatic β cells by interaction with SNARE proteins. *FASEB Journal, 2019; 33(11):12425-34. Doi: 10.1096 /fj. 201901007R.*
* equal contribution

III. **Golec E**, Ekström A, Noga M, Hmeadi MO, Lund PE, Villoutreix BO, Krus U, Wozniak K, Korsgren O, Renström E, Barg S, King BC, Blom AM. Alternative splicing encodes novel intracellular CD59 isoforms which mediate insulin secretion and are downregulated in diabetic islets. *In press in PNAS Journal, MS #2021-20083R.*

IV. **Golec E**, Noga M, Villoutreix BO, King BC, Blom AM. Interaction of intracellular CD59 isoform IRIS-1 with DNA in pancreatic islets- a potential involvement in regulation of gene transcription. *Manuscript*

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V. King BC, Esguerra JLS, **Golec E**, Eliasson E, Kemper C, Blom AM. CD46 activation regulates miR-150-mediated control of GLUT1 expression and cytokine secretion in human $CD4^+$ T cells. *Journal of Immunology, 2016; 196(4):1636-45. Doi: 10.4049/ jimmunol.1500516.*

VI. **Golec E***, Lind L*, Qayyum M, Blom AM, King BC. The Noncoding RNA nc886 Regulates PKR Signaling and Cytokine Production in Human Cells. *Journal of Immunology, 2019; 202(1):131-41. Doi: 10.4049/ jimmunol.1701234.*
* equal contribution

Abbreviations

MASP-1/2	MBL-associated serine proteases 1 and 2
MBL	Mannose-binding lectin
MAC	Membrane attack complex
RCA	Regulators of complement activation
CCP	Complement control protein domains
TSR	Thrombospondin repeat modules
C4BP	C4b-binding protein
CR1	Complement receptor 1, or CD35
DAF	Decay-accelerating factor, or CD55
MCP	Membrane cofactor protein, or CD46
GPI anchor	Glycosylphosphatidylinositol anchor
ER	Endoplasmic reticulum
PLC	Phospholipase C
PNH	Paroxysmal nocturnal hemoglobinuria
PIG-A	Phosphatidylinositol glycan anchor biosynthesis class A
CIDP	Chronic inflammatory demyelinating polyneuropathy
SUSD4	Sushi-domain containing protein 4
CSMD1	Human CUB sushi multiple domains protein 1
Dusp26	Dual specificity phosphatase 26
NNCIT	Nordic network for clinical islet transplantation
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
LADA	Latent autoimmune diabetes in adults
MODY	Maturity-onset diabetes of the young
NDM	Neonatal diabetes mellitus
SIRD	Severe insulin resistant diabetes
MOD	Mild obesity-related diabetes

MARD	Mild age-related diabetes
SIDD	Severe insulin deficient diabetes
GAD65	65 kDa glutamic acid decarboxylase
IA-2	Insulinoma-associated protein 2
ZNT8	Zinc transporter 8
LC3	Protein 1 light chain 3
IAPP	Islet amyloid polypeptide, or Amylin
PC1/3	Prohormone convertase 1/3
PC2	Prohormone convertase 2
CPE	Carboxypeptidase E
HLA	Human leukocyte antigen
LDCV	Large dense core vesicles
RRP	Readily releasable pool
SNARE	Soluble NSF attachment protein receptor
NSF	N-ethylmaleimide sensitive factor
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GCK	Glucokinase
SUR	Sulfonylurea receptor subunit
VDCCs	Voltage-dependent calcium (Ca^{2+}) channels
HNF1α	Hepatocyte nuclear factor 1α
HNF4α	Hepatocyte nuclear factor 4α
FPG	Fasting plasma glucose
OGTT	Oral glucose tolerance test
HbA1c	Glycated hemoglobin A1c
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
NGT	Normal glucose tolerance
ROS	Reactive oxygen species
FFA	Free fatty acids
UPR	Unfolded protein response
IL1-R	IL1-receptor
DAMPs	Danger-associated molecular patterns

PAMPs	Pathogen-associated molecular patterns
AGEs	Advanced glycation end products
ATG16L1	Autophagy related 16 like 1
IL-1β	Interleukin-1β
MAVS	Mitochondrial antiviral-signaling protein
UCN3	Urocortin 3
PDX1	Pancreatic duodenal homebox-1
hTERT	Human telomerase reverse transcriptase
SV40LT	Simian vacuolating virus 40 large T antigen
PI3P	Phosphatidylinositol 3 phosphate
mTORC1	Mammalian target of rapamycin complex 1
DSBs	Double-stranded breaks
HDR	Homology-directed repair
NHEJ	Non-homologous end joining
PLA	Proximity-ligation assay

Preface

Through millions of years of evolution, single cells developed into complex organisms. In order to function as a multicellular system, cells had to develop specialized functions, such as transport of nutrients, waste elimination, and protection of the organism from invading microbes. To fulfill these needs the circulatory system, which connects the body's parts, evolved.

The complement system is a substantial component of innate immunity, operating locally and in circulation. Its main functions are to dispose of pathogens, cellular debris, and apoptotic cells to ensure the organism's protection and homeostasis (1). These functions of complement are today well established. However, recent studies have broadened our understanding of the roles of complement and shifted our perspective on its functions. Instead of the older view of complement as acting almost exclusively extracellularly, new evidence points to its considerable roles in the basic processes in the cell interior. Such complement functions should not be surprising since primitive complement proteins have been identified in organisms as early as sponges (*Porifera*) (2), thus indicating that the complement system's origin is ancient and dates to over 500 million years ago. Sponges are the world's simplest, multicellular organisms lacking true organs or circulatory system. Therefore, it is tempting to suggest that complement evolved from a simple system involved in basic processes in a cell interior to the modern-day network acting in the blood.

The complement system

Complement was discovered over a century ago as a heat-labile blood component capable of killing bacteria and lysing erythrocytes from other species. In 1891 Buchner discovered that serum heated at 55 °C for 30 min lost its bactericidal property. He named this heat labile component of serum "alexin" in Greek "to ward off". Later, in 1895, Bordet supported this theory and demonstrated that alexin was capable of lysing erythrocytes (3). It was however Ehrlich who elucidated the mechanism of erythrocyte lysis by heat-stable anti-toxin (now known as antibody) and the heat-labile component of blood, which "complemented" the effect of immune serum (now known as complement) (4). This was the basis of the discovery of the classical pathway of complement. In 1908 Ehrlich and Metchnikoff (who discovered phagocytosis by macrophages) were jointly awarded the Nobel Prize in recognition of their work on immunity (4).

Since then, the perception of the roles of complement has markedly developed. Up to date, we recognize over 30 complement proteins that are found as inactive precursors circulating in the plasma, or embedded on cell membranes, functioning as complement receptors or regulators. However, the role of complement is not limited to protection against pathogens. Activated complement also marks target cells/ cellular debris for phagocytosis and induces inflammation through the generation of anaphylatoxins. Recently, the view of the complement system is evolving, discoveries introducing non-canonical roles of this system are made, and this seemingly simple system continues to surprise.

Activation of complement

Depending on the molecular trigger, the complement system can be activated via three distinct pathways. This system is activated through a triggered protein cascade, where active complement protein generated by cleavage of its precursor subsequently cleaves its substrate, another complement protein, to its active form.

In this way, the initial activation of a few complement proteins generates an amplification signal resulting in a large complement response. Therefore, tight regulation of these enzymatic cascades is essential to prevent the rapid consumption of complement in response to trivial stimuli and to prevent uncontrolled or insufficient complement activation.

Classical Pathway

The classical pathway of complement activation was the first described pathway, dating back over a century ago to the discovery of Ehrlich of the interaction between antibodies and the then-undescribed components of serum.

The prototypic activator of the classical pathway is indeed an antibody bound to the antigen. However, it is now known that many other molecules can initiate the classical pathway without the need for antibodies. These include gram-negative bacterial lipopolysaccharide, β-amyloid peptide aggregates, nucleic acids, apoptotic cells, and other structures (5). The central recognition unit of the classical pathway is the hetero-oligomeric C1 complex (6, 7). The C1 complex, a dynamic structure that constantly dissociates and re-associates in the circulation, consists of one molecule of a recognition subunit: C1q and serine proteases: C1r and C1s (two molecules of each) associated non-covalently in a Ca^{2+} dependent complex (8). The C1q molecule has a characteristic "bouquet of flowers-like" structure with a collagen-like bundle at the N-terminal and six globular heads at the C-terminus. The binding of C1q to an activator occurs via globular heads. The multivalent binding of hexameric C1q to the activator is Ca^{2+} dependent and inhibited by divalent cation chelators. C1q engaged with an activator undergoes conformational changes triggering the autoactivation of C1r. Activated C1r then cleaves and activates C1s. Activated C1s cleave C4 to C4a and C4b (9). C4a has antimicrobial activity (10), whereas C4b covalently binds to the complement-activating surface via its reactive thioester bond (11). Membrane-bound C4b express a binding site, which in the presence of Mg^{2+} ions binds C2 and presents it for cleavage by C1s into C2a and smaller fragment C2b. In general, complement cleavage fragments are named with letters according to their relative size, with "a" fragments smaller than "b" fragments. For the C2, however, the smaller fragment is often referred to as "C2b". Throughout this thesis, the conventional C2 nomenclature will be used. However, it is important to point out this matter and, in the future, adhere to the new recommendations for complement nomenclature

presented by the complement field (12). The smaller C2b fragment is released, whereas the C2a fragment remains attached to C4b, forming the classical pathway C3 convertase (C4b2a) (13). This C3 convertase cleaves the C3 molecule into C3a and C3b. Generated C3b binds to C3 convertase to form C5 convertase: C4bC2aC3b (classical and lectin pathway) or C3bBbC3b (alternative pathway). C5 convertase cleaves the C5 protein into C5a (small anaphylatoxin) and C5b- the first component of the membrane attack complex. Further steps of the cascade are described in a later section (terminal pathway).

Lectin Pathway

The lectin pathway is an antibody-independent way of complement activation. However, the molecules involved in this pathway activation resemble those of the classical pathway in many ways.

Activation of the lectin pathway starts with mannose-binding lectin (MBL), ficolins, or collectins (proteins similar to C1q with collagen stalks and carbohydrate-binding domains), which bind mannose and N-acetyl glucosamine (polysaccharides) present in abundance on bacteria, fungi and other pathogens(14).

MBL, ficolins, and collectins associate in Ca^{2+} dependent complex with MBL-associated serine proteases (MASP-1 and MASP-2), analogues to C1r and C1s. MBL, ficolins, and collectins found in plasma are mainly associated with MASP-1 and MASP-2 (15). Upon MBL, ficolins, and collectins binding to an activator, a conformational change leads to autoactivation of MASP-1, which in turn activates MASP-2. MASP-1 activation of MASP-2 can occur if both these proteins are in the same complex, but also where they are located on adjacent complexes (16). Both MASP-1 and MASP-2 cleave C2, whereas only MASP-2 can cleave C4. Once C4 and C2 are cleaved, a C3 convertase (C4b2a)- identical to that of the classical pathway is formed. Subsequent activation steps are identical to the classical pathway.

Three homologs of MASP-1 and MASP-2 have been identified: MASP-3, Map44, and Map19. They lack their C2 and C4 cleaving activity and are considered negative regulators of the lectin pathway, leading to its inhibition (17, 18).

Alternative Pathway

The alternative pathway is a pathway that is always active at very low levels and, as such, is primed to respond to infections rapidly. C3 is the crucial component of the alternative pathway; however, three other proteins are also required: factor B, factor D, and properdin (although the last one is required depending on the activator) (19).

C3b bound via a thioester bond to activating surfaces binds factor B (a 93-kDa plasma protein) in an Mg^{2+}-dependent manner. Once factor B is bound to C3b, it becomes susceptible to cleavage by factor D, a serine protease present in its active form in the plasma. Factor D, also called adipsin, is not made in the liver but is synthesized almost exclusively by fat tissues (adipocytes) (20, 21).

Adipsin cleaves factor B at a single site, releasing a 30 kDa fragment, Ba, and exposing a serine protease domain on the 60 kDa fragment Bb. Adipsin can cleave only factor B, when it is bound to C3b (20, 22). The C3bBb complex formed is the C3 convertase of the alternative pathway and cleaves C3 at a site identical to the one utilized by C4b2a from the classical pathway. Properdin binds and stabilizes the C3bBb complex, extending its lifetime by 5-10 folds (23). Properdin is a glycoprotein made up of oligomers formed by non-covalent interactions between monomers, each composed of six tandemly arranged repeats, called thrombospondin repeat modules (TSR) (24, 25). Alternative pathway initiation requires C3b to be already present on the surfaces in the conformation allowing adipsin to bind and cleave factor B. Thus, limited activation of the classical pathway (by depositing C3b) may trigger activation of the alternative pathway. Therefore, the alternative pathway acts as an amplifier of the classical pathway.

The alternative pathway can also be initiated independently of the classical pathway, as C3 is continuously hydrolysed at a slow rate and forms a $C3(H_2O)$ molecule. $C3(H_2O)$ binds factor B in the solution and makes it susceptible to adipsin cleavage. As a result, a C3 convertase ($C3(H_2O)Bb$) is formed and cleaves C3 into C3a and C3b. Each newly generated C3b will then deposit on self and foreign surfaces in the body and start the formation of a new convertase, leading to amplification of the alternative pathway activation. This phenomenon is known as “tick-over” and is used to rapidly neutralize pathogens (22).

Terminal Pathway

The terminal pathway starts when C5 is cleaved by C5 convertase to C5a and C5b.

C5b undergoes the conformational changes that allow it to associate with C6. The binding of C6 stabilizes the membrane-binding site in C5b and leads to exposure of the C7 binding site. If the C5b67 complex encounters a membrane, it associates tightly with the membrane of a pathogen or host cell (26, 27). At this stage, the complex cannot yet penetrate the membrane deeply and does not disturb its integrity. Next, the β chain of C8 (C8 is made up of three chains: α, β, δ) binds to C7 in the C5b67 complex resulting in a formation of the C5b-8 complex that becomes more deeply buried in the membrane, forming small pores, and causing the cell to become slightly leaky. At this point, the final terminal pathway component, C9, will bind. The first C9 molecule binds to C8α and undergoes a conformational change from a globular to an elongated form. The unfolding of C9 leads to its insertion in the membrane and the beginning of pore formation, as well as to the exposure of the binding site for additional C9 molecules. The membrane pore formation begins with a single C9 molecule. However, the membrane attack complex (MAC) may contain as many as 22 C9 molecules (26, 27).

Inserting MAC into the membrane bilayer forms functional pores in the membrane through which ions and small molecules pass, causing osmotic lysis of the cell. The terminal pathway is regulated at the level of MAC complex formation by the membrane-bound inhibitor, CD59 (detailed later in this thesis). However, it was shown that MAC can also be endocytosed in the case of MAC insertion into eukaryotic cells (28).

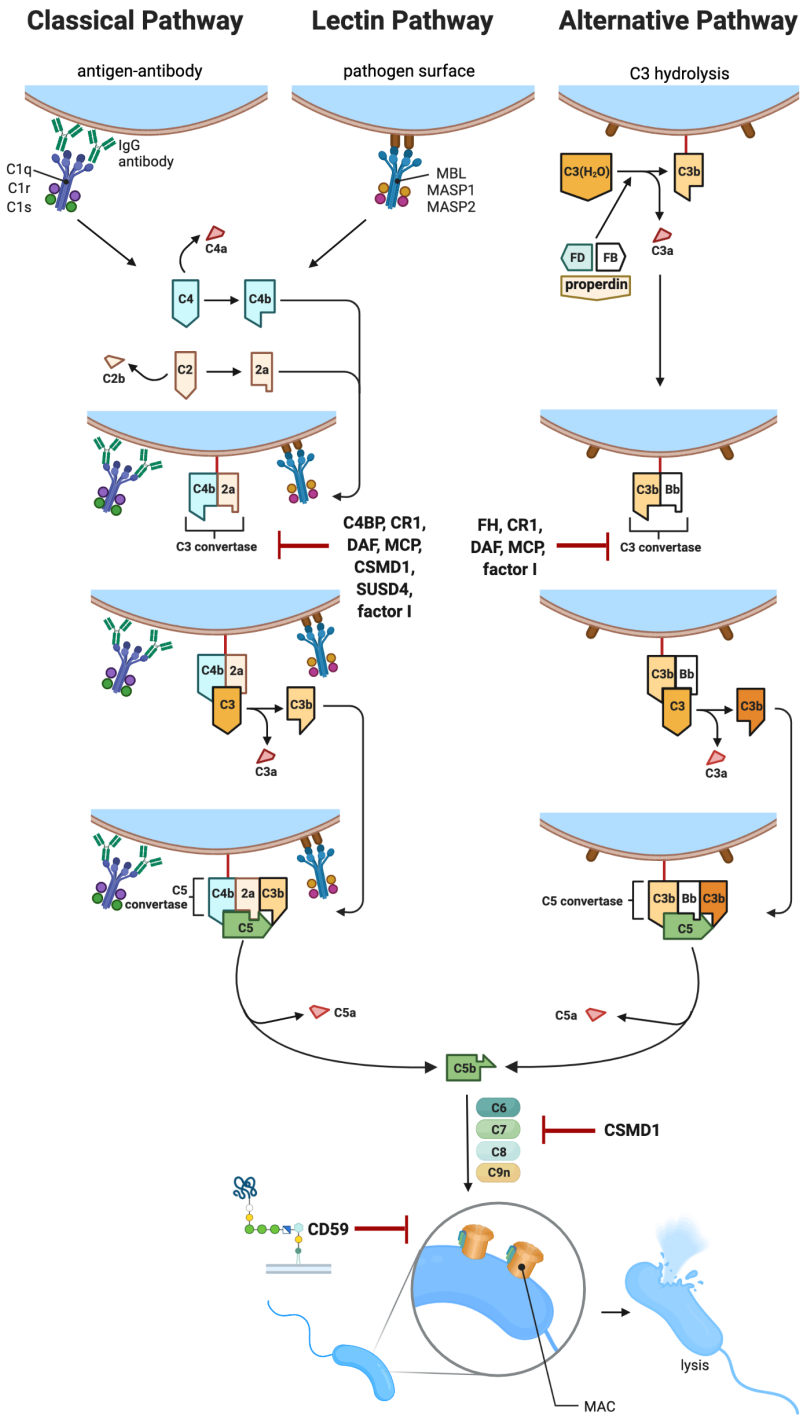

Figure 1. Simplified complement activation pathways.

C3-the central component of the complement cascade

C3 is one of the oldest and most important complement system proteins. It is abundantly present in serum in concentrations varying between 1-1.5 mg/ml. C3 is an acute-phase protein, and its serum levels rise approximately two-fold during infections. The hepatocytes primarily synthesize C3 in the liver (distributed intravascularly), but nearly every cell in the body can produce it (29).

C3 is a large glycoprotein (185 kDa) encoded by the C3 gene on chromosome 19. It is composed of two polypeptide chains: α (110 kDa) and β (75 kDa), linked via a single disulphide bridge (30, 31). Prior to secretion, a single polypeptide chain C3 precursor, pro-C3, is cleaved intracellularly by furin (in a Ca^{2+} dependent manner) to its mature form (32). C3 contains two main glycosylation sites at Asn-917 of the α-chain and Asn-63 of the β-chain (30, 33).

C3 undergoes a series of proteolytic cleavages upon complement activation through each pathway. C3 binds non-covalently to C2a from the C4b2a classical and lectin pathways convertase. It is then cleaved by the C2a at a single site in the α chain, releasing the C3a fragment (9 kDa) and inducing a conformational change in the remaining C3b, exposing a highly reactive thioester bond on the α chain of this molecule. C3 can also be cleaved by multiple serine proteases like thrombin (present in the blood) or cathepsin (cell-derived). Tick-over mechanism leading to spontaneous hydrolysis of the thioester bond on C3 results in formation of $C3(H_2O)$, allowing for factor B binding and adipsin cleavage, resulting in a fluid-phase C3 convertase ($C3(H_2O)Bb$), which can cleave additional C3 molecules. C3b is then attached via its highly reactive (but short-lived) thioester bond to the surfaces, creating an amplification loop for C3b deposition. Convertase formation can be prevented by cleaving C3b to its inactive form- iC3b (by factor I and cofactors). iC3b is unable to participate in the amplification loop formation but remains bound to the surfaces and enhance complement-mediated phagocytosis by binding to phagocytic receptors (CR3 and CR4). In contrast, C3b mediates

phagocytosis via the complement receptor of the immunoglobulin family (CRIg) (31).

As C3b is degraded into iC3b, the small C3f peptide is released. It was shown that C3f increases vascular permeability (34). However, not many functions are known for this peptide. The third cleavage site on iC3b produces C3c and C3dg. C3d is an end proteolytic product of C3dg cleavage.

C3a is a potent proinflammatory mediator: an anaphylatoxin. Anaphylatoxins trigger the degranulation of basophils and mast cells, resulting in the release of inflammatory mediators. C3a can also bind to its receptor (C3aR) on myeloid cells contributing to cellular activation and chemoattraction. However, recent reports suggested an anti-inflammatory role of C3a, which is supposed to reduce the mobilization of neutrophils into the circulation following injury (uninhibited mobilization can lead to an increase in neutrophil numbers at the injury site, worsening the disease progression). C3a can be quickly inactivated by cleavage at the C-terminal arginine to form C3a-desArg, which lacks its activity due to losing binding to C3aR (31, 35).

C3b is the best characterized C3 fragment. This protein not only amplifies the complement response via convertase formation but also acts as an opsonin to facilitate the clearance of tagged particles. C3b (coated on a pathogen) binds to complement receptor 1 (CR1), CD35 on phagocytes, enhancing pathogen engulfment and clearance. Also, tissue-resident macrophages, like Kupffer cells in the liver, bind C3b via the CRIg receptor to induce phagocytosis. C3b can also deposit on antigen-antibody complexes and make them easier to clear from the circulation. By this action, C3b blocks the formation of large, insoluble immune complexes that could accumulate in, e.g., small vessels leading to their damage.

Other roles of C3 and its cleavage products

Several groups suggested that C3a-desArg has metabolic hormone properties increasing glucose uptake and lipogenesis by adipocytes and skin fibroblasts, linking this molecule to the pathogenesis of obesity (enhancement of glucose and lipids storage in adipose tissues) (36-40). C3a was shown to act as a stimulator of insulin secretion from the β-cells of the pancreas. C3a, generated by alternative pathway activity, attributed to adipsin, binds to its receptor (C3aR) expressed on the surface of β-cells, leading to C3aR activation and an increased calcium influx

to the β-cells, potentiating insulin secretion (41-43). It was shown that adipsin (which is involved in adipose tissue homeostasis) levels are decreased in rodent models of diabetes and obesity and serum from T2D patients (42). In *db/db* mice (a model of human T2D), replenishment of adipsin improved glycemic status and decreased β-cell death. The combination of adipsin replenishment, and C3a treatment downregulated the expression of Dual Specificity Phosphatase 26 (Dusp26), which improved the β-cells function (44). It was shown that forced expression of Dusp26 in β-cell decreases the expression of β-cell identity genes and sensitizes the cells to death. Its inhibition improves hyperglycemia in diabetic mice and protects human islets from death (44).

C3a is also involved in central nervous system development: in neurogenesis and neuronal migration (45-48). Additionally, C3a promotes liver hepatocyte proliferation and regeneration after resection (49, 50). C3-knockout mice suffered increased injury after 70 % hepatectomy and increased steatosis- a condition in which excessive amounts of fat accumulate in hepatocytes due to failure in lipid metabolism. Administration of C3a-desArg to C3-knockout mice resulted in decreased steatosis and hepatic injury (51).

C3aR is also involved in providing survival signals for T-cells (52) by modulating mTOR activity (53).

C3b and iC3b are involved in synapses remodeling. C3b- and iC3b-mediated signaling promotes the engulfment of improper synaptic connections through recognition by C1q, therefore regulating brain development and memory formation (54, 55). Moreover, C3b and iC3b facilitate the removal of damaged cells from the liver, which was exposed to toxic carbon tetrachloride (49).

C3b also contributes to defense against viruses. C3b coated on the virus blocks it from binding to its receptor on host cells, preventing virus entry into the cell. C3 opsonized on pathogens upon their internalization activates mitochondrial antiviral-signaling protein (MAVS), resulting in proteasome-mediated clearance of viral particles (56). C3 directs pathogens that enter the cell (and have been previously extracellularly opsonized with C3) to the autophagy pathway (xenophagy) via interaction with autophagy-related 16 like 1 protein (ATG16L1), targeting them to destruction and limiting their intracellular growth (57).

C3 is involved in bone remodeling. Two main cell types are engaged in this process: osteoclast and osteoblasts. Osteoclasts are responsible for degrading aged bones, whereas osteoblasts for new bone formation. An activated form of vitamin

D (1 alpha 25 dihydroxy-vitamin D3) stimulates bone marrow cells to local C3 production and osteoclasts differentiation. Tu and colleagues presented that bone marrow cells produce C3 and other complement components needed to activate C3 and generate C3a and C5a, which signals via its receptors and regulate osteoclast differentiation (58, 59).

It has been suggested that C3 is involved in the differentiation of muscles. C3 is secreted by muscle-resident pre-adipocytes and is internalized by myogenic cells promoting their differentiation (60).

Resting T-cells contain endosomal and lysosomal C3 stores. Intracellular C3a is generated via cathepsin L cleavage (61). However, not only cathepsin L is able to cleave C3. It was shown that mast cells, long-living tissue-resident cells containing granules with inflammatory mediators (to fast mediate the inflammatory responses after infection) contain two major proteases: tryptase (62) and chymase (63, 64), which are able to cleave C3, producing its active fragments (62, 64).

Finally, it has been shown that in B cells, C3 is internalized and translocated into the nucleus, where it modulates the genes expression (65). Whether C3 has similar functions in other cell types remains to be addressed.

Regulators of the complement cascade

Complement system activation can have detrimental effects when occurring unnecessarily, uncontrollably, and on the wrong surface.

Autologous surfaces are protected from complement by both membrane-bound and soluble inhibitors. However, it was shown that numerous types of bacteria express or recruit complement inhibitors on their surfaces, allowing them to escape complement-mediated attack (66).

Many of the complement inhibitors are members of the RCA group (regulators of complement activation). The RCA proteins are composed of complement control protein domains (CCP), also known as sushi domains or short consensus repeats. Each CCP domain contains approximately 60 amino acids with two disulphide bonds between cysteine residues. The number of CCP domains in each regulatory protein varies from 2 to 37. The RCA proteins bind proteolytic fragments of C3 and C4 (67). There is also a small subset of regulators, which inhibit the formation of the terminal complement pathway. Inhibitors of the complement activation and terminal pathways are introduced below.

Regulatory proteins in the activation pathways

C1 inhibitor is a plasma protein, primarily synthesized in the liver, which rapidly inactivates C1 by forming a tight complex with activated C1s and C1r, resulting in a dissociation of these components from C1q, thus limiting the activation of the classical pathway (68).

Fluid phase RCA proteins include factor H and C4b-binding protein (C4BP). Factor H prevents the amplification of the alternative pathway by accelerating the dissociation of C3bBb, the alternative pathway convertase. Factor H is also a cofactor for the plasma serine protease Factor I. In the presence of factor H, factor I cleaves C3b at two sites, resulting in the release of C3f and enzymatically inactive iC3b (69, 70).

C4BP functions in the classical pathway, and its role is analogous to that of factor H in the alternative pathway. It accelerates the decay of the classical pathway convertases (C4b2a and C4b2a3b). C4BP is also a cofactor for factor I, promoting the cleavage and inactivation of C4b into iC4b, C4c and C4d (71).

Membrane-bound RCA proteins include complement receptor 1 (CR1) or CD35, decay-accelerating factor (DAF) or CD55, membrane cofactor protein (MCP) or CD46, sushi-domain containing protein 4 (SUSD4), and Human CUB Sushi multiple domains protein 1 (CSMD1).

Complement receptor 1 is involved in regulating both classical and alternative pathways. It acts as a decay-accelerating factor and a cofactor for factor I mediated breakdown of C3b and C4b. CR1 is the only cofactor that, under physiological conditions, mediates the cleavage of iC3b into C3c and C3dg (factor H can cleave iC3b only under conditions of low ionic strength), although the interaction between CR1 and iC3b is weak. The affinity of CR1 to C4b is smaller than for C3b. However, CR1 binds C4b in the C4b2a classical pathway convertase, leading to its decay and formation of inactive cleavage products: C4c and C4d (72).

DAF, as its name suggests, accelerates the decay of classical and alternative pathway C3 convertases: C4b2a and C3bBb, and the corresponding C5 convertases. DAF is linked to the membrane by a GPI-anchor; therefore, it can accelerate the decay of the convertases bound to the same membranes to which it is anchored. It cannot target the convertases present on other targets (73).

MCP acts as a cofactor for factor I mediated cleavage of C3b and C4b. MCP does not accelerate the decay of C3 convertase, and as such, it complements the action of DAF. Since MCP is a transmembrane protein, it only inactivates C3b bound to the same cell (74).

SUSD4 consists of two isoforms, SUSD4a and SUSD4b. SUSD4 inhibits the deposition of C4b and C3b, which are triggered by classical and lectin pathways. In the presence of SUSD4, the formation of classical pathway C3 convertase is inhibited, as C2 is not efficiently cleaved. SUSD4 was shown to bind C1q – free and in C1 complex (75).

CSMD family consists of three transmembrane proteins: CSMD1, CSMD2, and CSMD3. CSMD1 is the most extensively studied. CSMD1 inhibits the classical pathway and serves as a cofactor in factor I mediated cleavage of C4b and C3b. Additionally, CSMD1 inhibits the formation of MAC at the level of C7 (75).

Finally, factor I inhibits all pathways by cleaving C3b and C4b. Factor I in the circulation is proteolytically inactive and achieves its enzymatic abilities in the presence of cofactors, such as factor H (for C3b), CD46, CD35, and C4BP (76).

CD59- the regulatory protein in the terminal pathway

CD59 is a GPI-anchored membrane protein, able to inhibit the formation of the membrane attack complex (MAC), a multi-molecular assembly of complement proteins C5-to C9 that forms during the complement system activation.

During the first stage of MAC assembly (C5b-7 stage), a labile binding site on C7 is exposed, allowing MAC to bind to cell membranes. Further C8 and multiple C9 copies are incorporated into the complex, leading to the formation of a pore through the membrane bilayer and subsequent cell lysis. In the presence of CD59, the initial interaction of C9 with C5b-8 occurs; however, CD59 acts as a 'suicide inhibitor' locking itself onto C8 (CD59 is binding to the α-chain of C8 and the C-terminal b domain of C9), preventing the unfolding and insertion of multiple C9 into the complex, thereby blocking the cell lysis (77, 78). The association of CD59 in the complex is strong enough to withstand detergent extraction of the membrane. The amino acids involved in this interaction have been identified and cluster around the W65 site (79). Studies showed that deglycosylated CD59 retains its ability to inhibit complement-mediated cell lysis (80, 81).

The complement-inhibitory function of membrane-bound CD59 is markedly reduced in diabetes. Hyperglycaemia (high blood glucose levels) leads to non-enzymatic glycation of the Lys41 residue within the active site of CD59, resulting in its inactivation (82). Inactivation of CD59 results in the insertion of MAC into cell membranes, leading to the release of cytokines that promote inflammation and thrombosis seen in target organs of diabetic complications. Moreover, glycated CD59 serves as a biomarker of blood glucose handling (82, 83).

Structure and physicochemical properties

CD59 is a single domain protein (related to the Ly6/uPAR superfamily of cysteine-rich proteins) comprised of three β-sheets and one α-helix stabilized by five disulphide bonds (84). The gene encoding human CD59 is located at chromosome 11 (Chr11p14-p13) (85).

The immature CD59 protein is synthesized as a 128 amino acid single-chain precursor, including an N-terminus localization sequence of 25 amino acids and a 26 amino acids C-terminal sequence that includes the motif for glycosylphosphatidylinositol (GPI) anchor addition. CD59 becomes translocated into the endoplasmic reticulum (ER), where the N-terminal signal peptide is removed, and GPI transamidase mediates the anchor attachment to residue N102 concurrent with the removal of the C-terminus. The presence of a GPI anchor on CD59 was initially confirmed by releasing the protein from the cell surfaces by treatment with phosphatidylinositol-specific phospholipase C. The mature CD59 protein comprises 77 amino acids.

CD59 is an 18-23 kDa GPI-anchored membrane glycoprotein with a single, large (4-6 kDa) N-linked carbohydrate group on N43 (86). The protein is also predicted to be O-glycosylated on T76 and T77. After enzymatic deglycosylation, CD59 runs on SDS-PAGE as a band of molecular weight 12 kDa, indicating that the protein is heavily glycosylated and that the broad molecular weight range is due to its variable glycosylation (87).

Tissue distribution

CD59 is broadly and abundantly expressed. It is present on all circulating cells, endothelium, and most cells so far examined (88). Cell surface distribution of CD59 is patchy, reflecting its predisposition to occupy the lipid rafts. Erythrocytes express around 25 000 copies of CD59 per cell (89).

On all cell types examined so far, CD59 is GPI-anchored. However, the degree to which the GPI anchor is cleaved by phospholipase C (PLC) varies between the cells. It was shown that erythrocyte-bound CD59 is resistant to PLC cleavage (90). A soluble form of CD59 is found in urine at a 1-4 μg/ml concentration. This form of CD59 cannot incorporate into the membranes due to GPI anchor loss (91).

Other roles of CD59

CD59 has been described as an alternative ligand for CD2 on T-cells, binding to a site on CD2 distinct from the site utilized by CD58- adhesion molecule, implicating CD59 in T-cells activation (92, 93). However others have been unable to demonstrate the binding between purified or recombinant CD2 and CD59,

making this an area of controversy. CD59 is also 'pirated' for use as a receptor for the pore-forming toxin intermedilysin. Intermedilysin is a toxin secreted by the gram-positive bacterium *Streptococcus intermedius*, able to create pores in the membrane of human erythrocytes through binding to CD59 (94). Increased expression of CD59 was found on several tumour cell lines and solid tumours (95, 96), suggesting that CD59 overexpression protects the tumour from complement attack. On the contrary, leukemic cell lines have little or no CD59 expression and, despite this deficit, continue to proliferate (97).

CD59 deficiency

The most common disease associated with deficiencies in membrane regulators is paroxysmal nocturnal hemoglobinuria (PNH), in which the synthesis of the glycosylphosphatidylinositol (GPI) anchor is defective.

PNH is a very rare disease, with a prevalence of 15.9 cases per million in Europe, which is caused by a mutation in the gene encoding the enzyme essential for GPI anchor synthesis: phosphatidylinositol glycan anchor biosynthesis class A (PIG-A) on hemopoietic stem cells. PIG-A encodes a GPI biosynthesis protein, phosphatidylinositol N-acetylglucosaminyltransferase subunit A. Different mutations in the PIG-A gene have been found, but all result in failure of GPI anchor synthesis. In the majority of the cases, PIG-A mutations arise *de novo*.

Because the mutation affects hematopoietic stem cells and due to the pluripotent nature of the stem cells, this mutation affects all the blood cells, including erythrocytes, platelets, and leucocytes. The percentage of circulating cells derived from the PNH clone varies from a few percent to nearly all cells.

The list of proteins anchored via GPI anchor and absent in PNH patients is long. However, there are two complement activation regulators: CD59, and DAF. The absence of these proteins makes all the circulating cells susceptible to complement-mediated lysis. Due to the lack of GPI-bound complement inhibitors on the surface, erythrocytes are being lysed by the complement system.

The clinical manifestation of PNH is hemolysis, causing hemoglobinuria, which is primarily noticeable in the morning, hence the name of the disease. Platelets and neutrophils that lack DAF and CD59 are more resistant to complement-mediated lysis than erythrocytes but are still being damaged, leading to its activation and increased adhesiveness. Sticky platelets and neutrophils will form micro-thrombi,

blocking small blood vessels in the brain, lungs, and kidneys. Therefore, strokes are the primary cause of morbidity in PNH patients.

PNH is treated with eculizumab, a monoclonal antibody that binds to the complement protein C5 and prevents the terminal complement pathway activation and subsequent intravascular haemolysis. The use of eculizumab has revolutionized the treatment of patients with PNH disease, and those patients now have a life expectancy comparable to the normal population (98). Additionally, the risk of thrombosis has been markedly reduced (85 % relative risk reduction) with eculizumab (99).

In patients with homozygous Cys89Tyr CD59 mutation (amino acid substitution p. Cys89Tyr), CD59 loses its function. This mutation was found in several patients aged 1-4.5 years, members of four unrelated families of North African origin. Nevo and colleagues observed that the Cys89Tyr mutation is not interfering with CD59 biosynthesis but perturbs its transport and localization to the cell surface (100). The authors found that patients carrying this mutation had normal expression of CD55. Since the Cys89Tyr mutation in CD59 is associated with the failure of CD59 to localize to the cell surface, this mutation was clinically manifested by recurrent strokes, chronic hemolysis, and a relapsing peripheral demyelinating disease resembling recurrent Guillain-Barre syndrome (GBS) or chronic inflammatory demyelinating polyneuropathy (CIDP). These results suggest that CD59 deficit is associated with demyelination via MAC activation. Additionally, the accumulation of unprocessed CD59 in neural cells can cause endoplasmic reticulum stress, which can be responsible for the part of the pathogenesis (100).

CD59 deficiency is common in patients with PNH. However, in PNH, the PIGA mutation affects only hematopoietic cells, whereas the Cys89Tyr mutation appears in all cells of the body (101). As a result, patients with Cys89Tyr mutation suffer from recurring GBS or CIDP-like disease, attributed to the demyelination in the peripheral nervous system via MAC activation, which is not seen in PNH. Patients with Cys89Tyr CD59 mutation are treated with eculizumab (102). This treatment showed marked clinical improvement, halted the patient's neurological deterioration, and reversed some motor handicaps, suggesting that eculizumab may be a life-saving treatment for patients with this mutation (102)

An unrelated family with the same clinical manifestations had a homozygous D49V mutation on the CD59 gene. No surface CD59 expression was detected (103).

Diabetes mellitus-classification

Type 1 diabetes mellitus (destruction of β-cells, usually leading to absolute insulin deficiency)

Type 1 diabetes mellitus (T1DM), also known as autoimmune diabetes, insulin-dependent diabetes or juvenile-onset diabetes, accounts for only 5-10 % of all diabetes cases and affects children primarily; however, this disease can develop in adults as well (104).

T1D is a chronic autoimmune disease that is characterized by hyperglycaemia (increased blood glucose level) due to insulin deficiency that occurs as the consequence of autoimmune destruction of the pancreatic β-cells. Markers of the immune destruction of the β-cells include several types of autoantibodies: autoantibodies to insulin, to 65 kDa glutamic acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2, IA-2β), and zinc transporter 8 (ZNT8). One or combination of these autoantibodies is detected in patients usually many months before symptom onset. These autoantibodies are not thought to be pathogenic but serve as biomarkers of autoimmunity (104-106).

The pathogenesis of the disease is thought to involve β-cell auto-antigen presentation by B and dendritic cells and the subsequent activation of β-cell-specific T-cells (auto-antigen-specific $CD4^+$ and $CD8^+$ T-cells).

The triggering event prompting β-cells autoimmunity is unknown. However, studies proved that the greatest contribution to the genetic susceptibility of T1D is exerted by histocompatibility antigens (HLA) class II alleles on chromosome 6: HLA-DQ haplotypes DQ2 and DQ8. HLA-DQ6.2 is negatively associated (protective) against T1D (107-109). DQ heterodimers present antigenic peptides to the immune system, but the mechanism by which DQ2 and DQ8 increase the risk for T1D is not fully understood. Almost 90 % of children newly diagnosed with T1D carry DQ2/8 haplotypes (110), and several investigations suggest that HLA contributes to about 60 % of the genetic risk of T1D (111). Therefore, considerable efforts have been made to identify non-HLA genetic risk factors for

this disease. Studies found more than 40 genetic factors associated with a high risk for T1D, among them polymorphisms within *PTPN22*, *CTLA4*, and *INS VNTR* genes, which can possibly contribute to higher T-cells activation (112).

Other studies suggest that cytotoxic T-cells specific to viral antigens are able to kill β-cells (113, 114). However, these studies do not provide an answer as to what extent virus infected β-cell is co-presenting viral and self antigens on MHC class I molecules making the β-cells a target for cytotoxic T-cells. Viral replication in β-cells may also result in β-cells apoptosis or necrosis. Finally, Coxsackie B3 virus was found in β-cells isolated from T1D new-onset patients (115).

Type 2 diabetes mellitus

The pathophysiology of type 2 diabetes (T2D) has been described in detail in later sections of this thesis.

However, it is important to mention that T2D is an insidious disease that develops after years of pre-diabetes. High glucose levels and metabolic and inflammatory stress slowly impair insulin secretion, which goes unnoticed in the early stages. An affected individual can live with the disease for many years without knowing of it, while important pathophysiological and perhaps irreversible changes occur in the body. It is estimated that approximately 46 % of all diabetes cases in adults are undiagnosed, and those individuals do not receive treatment that could reduce the risk of complications and premature death (116). The prolonged, asymptomatic phase of T2D (unmanaged high blood glucose levels lasting years) may lead to the development of micro and macro-vascular complications. Macro-vascular complications affect large blood vessels and are associated with the development of coronary heart disease, stroke, and peripheral vascular disease. Micro-vascular complications affecting small vessels may induce retinopathy, neuropathy, and nephropathy.

Even though proper treatment reduces diabetes-associated morbidity and mortality, patients with diagnosed T2D receiving treatment are still more likely to develop cardiovascular disease and have decreased life expectancy compared to non-diabetic individuals (117). According to the International Diabetes Federation, around 537 million people had diabetes in 2021, and the number of people living with diabetes is estimated to rise to 643 million by 2030 (https://diabetesatlas.org).

Moreover, just in 2021 diabetes contributed to 6.7 million deaths (1 every 5 seconds).

Ahlqvist and colleagues suggested novel subgroups of T2D, superior to the conventional diabetes classification (118). They showed that individuals with severe insulin-resistant diabetes (SIRD subgroup) and high BMI had a greater risk of diabetic kidney disease than individuals with mild-obesity-related diabetes (MOD cluster) or mild-age-related diabetes (MARD cluster). Moreover, patients with severe insulin deficiency (SIDD cluster) and negative for GADA autoantibodies had the highest risk of developing retinopathy among all the subgroups. All of these patients, however, had been prescribed similar diabetes treatment. These data suggest that the new clustering of patients with T2D might be helpful in guiding the right therapy for the patient's benefit (118).

Taken together, more measures are needed to prevent the increase in T2D and recognize the exact mechanism underlying the disease in order to improve the treatment to avoid complications and premature death.

Latent autoimmune diabetes in adults (LADA)

In 1977 Irvine et al. described that 11 % of patients initially diagnosed with T2D had antibodies against β-cells, which is characteristic of T1D. This separate subgroup of diabetes is called latent autoimmune diabetes in adults (LADA) (119).

LADA accounts for 3-12 % of all diabetes cases in adults (120). Individuals with LADA share the same phenotypical features with T2D but immunological features with T1D. In the case of LADA, the autoimmune process is milder, and the progression of β-cells failure is slower than in patients with T1D. Also, patients with LADA display higher levels of C-peptide. Antibodies against glutamic acid decarboxylase (GADA) are most frequently found in LADA patients (90 % of the antibody-positive patients were positive for GADA) than other auto-antibodies (10 % of the antibody-positive patients could be detected with antibodies against insulinoma-associated protein 2 (IA-2, IA-2β) or zinc transporter 8 (ZNT8)). LADA patients also display an insulin resistance, but not as pronounced as in T2D (120).

Monogenic diabetes

Monogenic diabetes is relatively rare and occurs due to genetic defects in single genes in the β-cells of the pancreatic islets, which reduces the β-cells function or mass. Typically, monogenic diabetes is classified according to the age of onset and is divided into two major groups: Neonatal diabetes mellitus (NDM), and Maturity-onset diabetes of the young (MODY), which are described below.

Maturity-onset diabetes of the young (MODY)

Maturity-onset diabetes of the young (MODY) usually presents before the age of 25. Mutations and deletions in at least six genes are causes of most MODY cases.

Mutations in hepatocyte nuclear factors 1α and 4α (*HNF1* α, *HNF4*α) and glucokinase: Hexokinase IV (*GCK*) underline two most common forms of MODY. Reviewed in (121, 122).

Glucokinase is a predominant glucose-phosphorylating enzyme. More than 150 different mutations in *GCK* have been identified to cause MODY2. Mutations of *GCK* account for 30-50 % of MODY cases. These mutations impair the *GCK* enzymatic activity, resulting in a decrease in the glycolytic flux into β-cells of the pancreas. As a result, the glucose-sensing abilities of the β-cells are severely decreased, and the blood glucose threshold level, which triggers insulin secretion, is increased. Patients with MODY2 have mild fasting hyperglycemia (121, 122).

Other MODY forms are due to mutations in β-cells transcription factors. Mutations in hepatocyte nuclear factors 1α and 4α (*HNF1*α, *HNF4*α) account for a great part of MODY forms. Mutations in the *HNF4*α cause MODY1, whereas mutations in *HNF1*α cause MODY3. These two types of MODY display almost identical clinical characteristics. In contrast to MODY2, MODY1 and 3 are more severe forms of diabetes, often evolving towards insulin dependency. The hepatocyte nuclear factors are expressed in various tissues, including the liver, pancreas, and kidney. *HNF1*α is a transcription factor essential for the development and function of the pancreas, as it regulates the expression of crucial β-cells genes, like GLUT2 or insulin. The function of β-cells is deteriorated due to their inability to increase insulin secretion in response to hyperglycemia. The *HNF1*α deficient mice have severe dysfunction of hepatocytes, renal tubular cells, and β-cells and develop diabetes (121, 122).

Finally, a deletion in the pancreatic duodenal homebox-1 (*PDX1*) coding region (required for β-cells differentiation) was also found in patients with MODY. Patients with homozygous mutations in *PDX1* are rare, develop pancreatic agenesis (partial or total loss of the body and tail of the pancreas), and suffer from diabetes (121, 122).

Neonatal diabetes mellitus (NDM)

Neonatal diabetes mellitus (NDM) presents within the first six months of life and can persist through life or disappear during infancy and reappear later in life.

In contrast to T1D, NDM is rarely associated with high-risk human leukocyte antigen (HLA) or the presence of islets autoantibodies.

There are over 20 known genetic causes of NDM. NDM is often due to monogenic defects- mutations in single genes. 40 % of patients with NDM have mutations in *KCNJ11* and *ABCC8* genes, affecting K_{ATP} channels (123).

Roles of β-cells in the regulation of blood glucose

β-cells in the islets of Langerhans

Insulin is a hormone secreted from endocrine mini-organs in the pancreas, called the islets of Langerhans, or pancreatic islets. The name islet of Langerhans was introduced in 1893 by Edouard Laguesse- a histologist from the University of Lille, who hypothesized that they are involved in endocrine secretion. He named them after Paul Langerhans, who described these cells clusters in his doctoral thesis in 1869, but he could not assign them a specific function (124).

The islets of Langerhans are ellipsoidal or spherical clusters of cells (approximately 1500 cells in each islet) with a mean diameter of 140 μm distributed within the exocrine pancreas (125). The total number of islets in a healthy human pancreas has been estimated to be between 3 to 15 million (126, 127). Adult human islets are composed of a mixture of five endocrine cell types: β-cells secreting insulin and amylin, α-cells secreting glucagon, δ-cells secreting somatostatin, PP-cells secreting pancreatic polypeptide, and ε-cells secreting ghrelin. The proportion of different endocrine cell types in the human islets varies considerably; however, adult human islets contain approximately 60 % β-cells, 30 % α-cells, and 10 % of the remaining endocrine cell types (δ, PP, and ε-cells), of which δ-cells are the majority (128, 129). Epsilon cells were the latest cell type discovered and represent approximately 1 % of the islet cells (130).

β-cells form the bulk of endocrine cell mass, and each β-cell contains approximately 9-13 000 secretory granules (131, 132). A thin collagen layer separates the pancreatic islets from the surrounding acini, and a dense network of capillaries penetrates each islet (133). Pancreatic islets are highly vascularized due to timely responses to changes in the plasma glucose levels and the release of hormones into the circulation, which is critical for adequate glucose homeostasis.

The degree of variation in islet composition differs between the species and may be important when considering results from rodent models of T2D. Rodents, such as mice, rats, hamsters, and guinea pigs, have round islets with glucagon, somatostatin, and PP-positive cells in the periphery and insulin-positive cells in the center of the islet (134). In comparison, it has been suggested that endocrine cells are distributed within the human islets without specific clustering (128, 129). Additionally, human islets contain a lower proportion of β-cells than mouse islets (129).

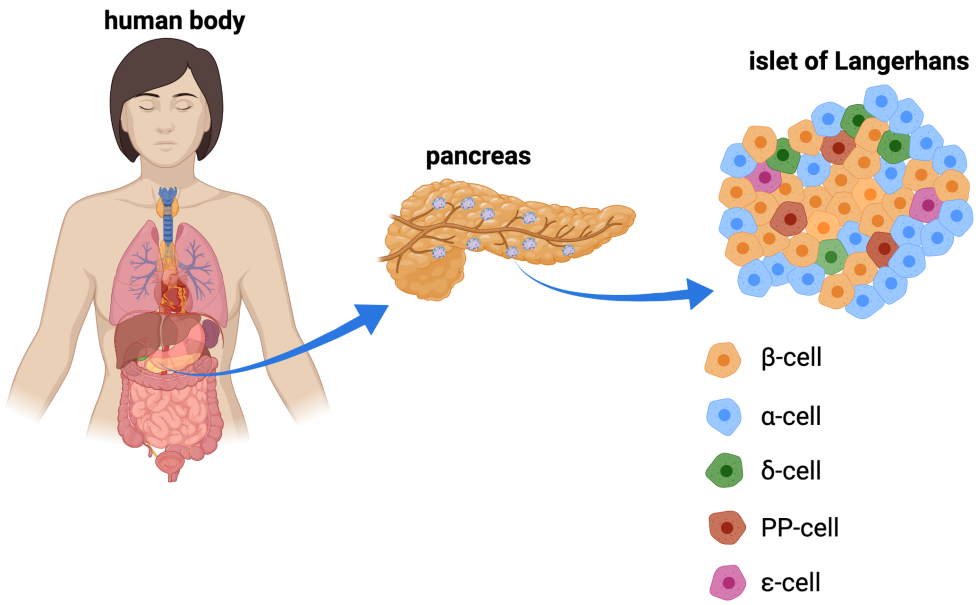

Figure 2. Schematic illustration of a human pancreatic islet.

Insulin processing

Like all peptide hormones, insulin is derived from a biologically inactive precursor molecule- preproinsulin. Insulin is the most abundant transcript in the β-cells, constituting approximately 10-15 % of the total mRNA. It was shown that glucose (in addition to stimulating insulin gene transcription and mRNA translation) enhances insulin mRNA stability. Reduced insulin mRNA stability was observed under low glucose concentrations, whereas it increases at high glucose levels (135).

During translation of insulin mRNA, signal peptide directs preproinsulin to the lumen of rough endoplasmic reticulum (ER). The hydrophobic N-terminal signal peptide of preproinsulin (110 amino acids) is cleaved off, resulting in proinsulin (81 amino acids). In the ER, proinsulin undergoes folding, and disulfide bonds are formed (136).

The precursor molecule is composed of three parts; A and B chains (21 and 30 amino acids, respectively) linked by three disulfide bonds (which stabilize the protein structure and are formed in the ER), and a C chain which is cleaved off in the Golgi apparatus and early secretory vesicles (136, 137). The folded proinsulin is transported to the Golgi apparatus, where insulin is packed into secretory vesicles. Secretory granules contain, except for insulin: proinsulin, C-peptide, IAPP, zinc, and other less abundant products like proteolytic enzymes (138). Islets amyloid polypeptide (IAPP), also known as amylin, is co-secreted with insulin from the β-cells. Under pathological conditions, IAPP polymerizes and forms large, intra-islet amyloid deposits- characteristic for T2D and insulinoma, which is described in detail later in this thesis.

The newly formed, immature insulin granules contain endo- and exo-peptidases, which process the insulin: prohormone convertase 1/3 and 2 (PC1/3, PC2) cleave off the C-peptide (31 amino acids), which is released together with insulin in a 1:1 molar ratio (137, 139). Additionally, carboxypeptidase E (CPE) removes the C-terminal residues from the B chain. Depending on the granule maturation stage, secretory granules may contain unprocessed proinsulin: this happens when the proteolytic enzymes present in the newly formed granule have not yet cleaved the precursor molecule.

Secretory granules in the β-cells undergo maturation in the cytoplasm. In order to become release-competent, secretory granules go through an ATP-dependent process called priming, which involves acidification of the granules via an ATP-dependent proton pump. The influx of positively charged protons is coupled with the influx of negatively charged chloride ions through chloride channels. This prevents the build-up of a large electrical gradient in the granule membrane (140). Studies have shown that elevated proinsulin levels are characteristic for T2D patients (141-145). Raised proinsulin levels in those patients may derive from compensatory hyperinsulinemia in insulin-resistant states and/ or inefficient proinsulin processing, indicating insulin secretory machinery defects.

Insulin is stored in cytoplasmic large dense-core vesicles (LDCV) with an electron-dense core and a clear peripheral coat (146). Each insulin granule has a

diameter of around 350 nm. Insulin (but not proinsulin) is complexed to zinc within the granules, forming insoluble insulin-zinc hexamers. Although the reason for zinc complexation is not well understood, its presence benefits islets isolation procedures (zinc-chelating dyes: dithizone, are used to determine islets purity) (147). The zinc-insulin crystals reside in the dense core of the secretory granule, whereas the soluble C-peptide often resides in the peripheral coat of the granules. Hexamers of insulin are the storage form of insulin and are secreted from the β-cells. However, upon secretion into the blood, they diffuse into monomers, which are the active form of insulin that binds to insulin receptors. It was shown that C-peptide enhances insulin action by speeding up the dissociation of insulin hexamers (148). Mutations in the insulin gene, affecting its biosynthesis and folding, lead to the neonatal-onset of diabetes mellitus (149).

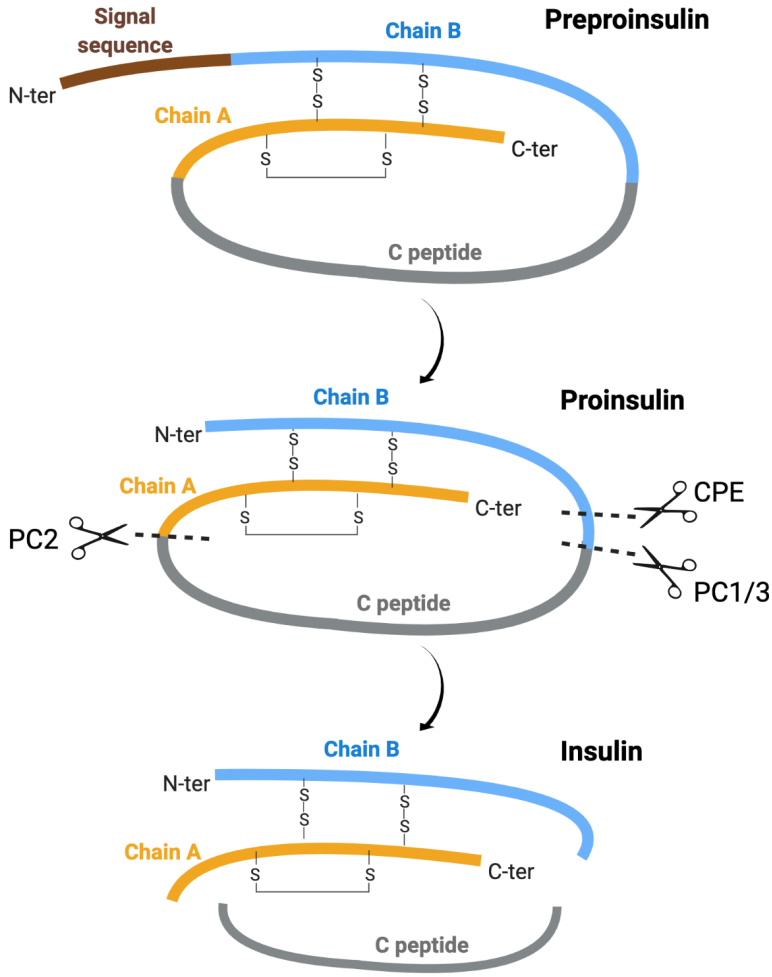

Figure 3. Scheme shows insulin processing by the prohormone convertases (PC1/3, PC2) and carboxypeptidase E (CPE) in the secretory granules.

Mechanism of insulin secretion from β-cells

Glucose enters the β-cell via GLUT1 (glucose transporter 1) and GLUT2 (glucose transporter 2). GLUT1 is a high-affinity low-capacity glucose transporter responsible for basal glucose uptake in most tissues. GLUT2 is a low-affinity, high-capacity glucose transporter accountable for glucose sensing (150).

β-cell secretes the insulin in response to glucose in a process called the stimulus-secretion coupling mechanism.

Raised plasma glucose levels result in glucose flux into the β-cell via a high-capacity, low-affinity GLUT2 transporter (150, 151). The cytosolic glucose is converted to glucose-6-phosphate by glucokinase (hexokinase IV). This process prevents the glucose from leaving the cell (152). Next, during the glycolysis (occurring in the cytosol), glucose-6-phosphate is converted to pyruvate. Further steps of glucose metabolism include the conversion of pyruvate to acetyl coenzyme A (acetyl-CoA), which enters the citric acid cycle. The main products of the citric acid cycle are CO_2, NADH, and $FADH_2$, which then undergo oxidative phosphorylation leading to adenosine triphosphate (ATP) production, which is transported out from the mitochondrial matrix. Both the citric acid cycle and oxidative phosphorylation occur in the mitochondria (153).

An increase in the cytosolic ATP level (ATP/ADP ratio) results in ATP binding to the regulatory Kir6.2 subunit of the ATP-sensitive potassium K_{ATP}-channels leading to channel closure. (K_{ATP} channel consists of four pore-forming subunits: Kir6.x and four regulatory sulfonylurea receptor subunits (SUR): SUR1 and Kir6.2 in islets) (154). K_{ATP} channels are responsible for maintaining a resting potential. Therefore, the closure of K_{ATP} channels due to increased cytosolic ATP levels will prevent the potassium from leaving the cell, leading to depolarization of the cell membrane, triggering the opening of voltage-dependent calcium Ca^{2+} channels (VDCCs). L-type (long-lasting) Ca^{2+} channels: $Ca_V1.2$ and $Ca_V1.3$ are primarily responsible for calcium-dependent insulin exocytosis from β-cells (154). The influx of Ca^{2+} through VDCCs channels results in the cytosolic calcium concentration elevation, which stimulates insulin secretion. Insulin exocytosis is triggered through Ca^{2+}-dependent insulin granule fusion with the plasma membrane. In order to mediate insulin exocytosis, several membrane-associated proteins are required.

The heart of exocytotic machinery is constituted by SNARE proteins (soluble N-ethylmaleimide sensitive factor (NSF)-attachment protein receptor), a family of over 35 small, primarily membrane-bound proteins (155). SNAREs vary in size and structure. However, they share a homologs sequence: the SNARE motif (155). Most SNAREs are attached to the target membrane via the C-terminal transmembrane domain, adjacent to the SNARE motif. These proteins associate to form a complex that bridges and fuses the membranes, and are dissociated again afterward, resulting in the SNARE cycle. SNARE proteins localize to vesicles and target membranes and are called v- and t-SNAREs. In the β-cells t-SNARE proteins: Syntaxin1 and SNAP25 localize to the cytoplasmic side of the plasma membrane, whereas v-SNARE: VAMP2, often called synaptobrevin associates

with insulin granules. Vesicles packed with cargo, e.g., insulin, are carried by motor proteins: myosin and kinesin families towards the plasma membrane through actin filaments and microtubules in a process fuelled by ATP (156). Membrane fusion results from the binding of the v-SNARE VAMP2 to the target t-SNARE: Syntaxin1 and SNAP25 (157). Upon membrane fusion, the SNARE proteins assemble into a four-helix bundle (the SNARE core complex) comprised of four parallel-orientated helices: one helix from VAMP2, one from Syntaxin1, and two helices contributed by SNAP25 (158). The SNARE motifs mediate the formation of the SNARE complex. SNARE proteins facilitate membrane fusion in a way analogous to the zipper. The formation of the SNARE complex begins from the membrane-distal N-terminal end of the SNARE motif and propagates towards the carboxyl-terminal membrane-proximal anchor. In this direction, the SNARE complex acts as a molecular zipper that pulls the membranes close together (159, 160). This process may provide the energy required to overcome the electrostatic forces between the vesicle and target membrane during fusion. In agreement with this theory is the finding that SNARE complexes are sufficient to catalyse the liposome fusion (161). The SNARE proteins play a role in membranes fusion and ensure that calcium influx is restricted to areas where secretory granules are in close contact with the plasma membrane. The intracellular loop of L-type Ca^{2+} channels (primarily $Ca_V1.2$) binds to Syntaxin1, SNAP25, and synaptotagmin, tethering the Ca^{2+} channel to the secretory granule. This complex is often referred to as the "synprint" region.

Synaptotagmins comprise a large group of proteins containing two Ca^{2+} binding sites: C2A and C2B, and they act as Ca^{2+} sensors in vesicle fusion. Different studies have shown that different isoforms of synaptotagmins: III, IV, V, VII, VIII are needed for mediating calcium-dependent secretion in β-cells (162-165). However, whether a single synaptotagmin isoform is required or whether they function in combination remains unclear (154).

Thanks to the "synprint" arrangement (Syntaxin1, SNAP25, and synaptotagmin bound to synprint site of L-type Ca^{2+} channel), the release-competent granules are exposed to high calcium levels, resulting in exocytosis of insulin. Calcium channels are themselves a part of the fusion machinery (166).

Recent studies have indicated that v- and t-SNAREs can be partially zippered but are prevented from fully assembling into a four-helical bundle by a protein termed complexin. The binding of calcium to synaptotagmin blocks the inhibitory

function of complexin, allowing for fusion to proceed. Thus, complexin seems to function as a repressor of membrane fusion (167, 168).

Secretory vesicles exist in distinct functional pools. Only a small fraction of the granules (1-5 %) belongs to the readily releasable pool (RRP). Granules belonging to this pool are release-competent or can quickly gain a release competence. The release of RRP of granules is believed to happen in the first phase of secretion (lasting between 5-10 min), and its end marks the depletion of this pool. The RRP granules are pre-docked to the plasma membrane by VAMP2, Syntaxin1, SNAP25, and synaptotagmin bound to the synprint site of the L-type Ca^{2+} channel (154, 169). The majority (95-99 %) of granules belong to a non-releasable reserve pool and must undergo priming (ATP and calcium-dependent process) to gain release competence (154, 169). Thus, insulin is secreted in a biphasic fashion, consisting of an initial, rapid 1st phase of secretion (preferable to the release of RRP of granules) and a subsequent 2nd phase of slower, prolonged secretion, presumably reflecting the low rate at which new granules from the reserve pool are supplied for release (154, 169).

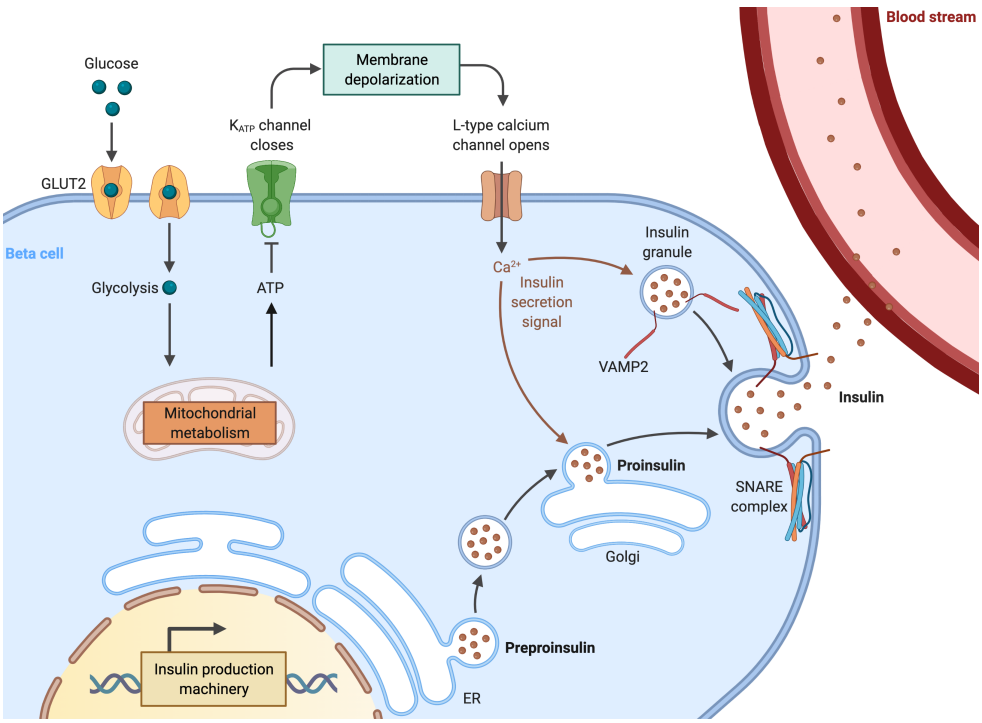

Figure 4. Stimulus-secretion coupling mechanism in the β-cells of the pancreas.

The course of type 2 diabetes mellitus

Definition and diagnosis of type 2 diabetes mellitus

T2D is diagnosed based on clinical tests measuring elevated glucose levels. These tests include a fasting plasma glucose test (FPG), oral glucose tolerance test (OGTT), and test measuring levels of glycated hemoglobin A1C (HbA1c). Hemoglobin A1C test reflects the average amount (percentage) of glucose bound to hemoglobin on red blood cells over the past two-three months (lifespan of red blood cells). A fasting plasma glucose levels above 7 mM/L, glucose level at 2 h during a 75 g OGTT above 11.1 mM/L, or HbA1c above 6.5 % are the criteria for T2D according to American Diabetes Association (170). Fasting plasma glucose between 5.6-6.9 mM/L is defined as impaired fasting glucose (IFG), glucose levels between 7.8-11 mM/L during the 2 h 75 g OGTT test are defined as impaired glucose tolerance (IGT). IFG, IGT, and HbA1c between 5.7 %-6.4 % are defined as prediabetes (170).

Pathophysiology of type 2 diabetes mellitus

T2D is a disease of two main factors: defective insulin exocytosis by pancreatic β-cells that is accompanied by insulin resistance- the inability of insulin-sensitive tissues to respond to insulin (171, 172). In predisposed individuals, T2D occurs when the adaptive capacity of the endocrine pancreas fails to meet the body's demands, and it is believed that the dysfunction of the β-cell is central to the development and progression of T2D.

A series of metabolic changes occur as an individual goes from normal glucose tolerance (27) to prediabetes and, finally, T2D manifestation (173). Reports suggest that insulin resistance in peripheral tissues is already well established before impaired glucose tolerance (IGT) develop (174). The first phase of insulin secretion is markedly reduced early during the T2D progression (175). In T2D patients fasting plasma glucose is approximately 7.8 mM/L or higher. However, it

was shown that the first phase of insulin secretion declines rapidly as fasting glucose levels rise beyond 5.6 mM/L and is completely loss above 6.4 mM/L (173). First phase insulin secretion is critical for controlling glucose levels, as it inhibits hepatic glucose production (175). Individuals with IGT and decreased first-phase insulin secretion had higher plasma glucose levels than those with normal glucose tolerance (NGT). It was caused entirely by impaired hepatic glucose production (48 % inhibition of hepatic glucose production in NGT vs. 28 % inhibition in IGT) (176). Additionally, above fasting glucose levels of 7.8 mM/L, the compensatory hypersecretion of insulin from the pancreatic β-cells starts to decline (174). Moreover, β-cells glucose sensitivity decreases drastically with increasing glucose intolerance in peripheral tissues (177).

Other than that, reduced functional β-cell mass (which is the mean of β-cells number, volume, and the ability of each β-cells to secrete insulin) has been reported in patients with T2D (178-180). It is generally assumed that the β-cell loss in T2D is due to enhanced β-cell apoptosis (178). Current evidence shows that the reduction in β-cells mass, which is visible at the early stages of the disease, seems insufficient to cause diabetes if not accompanied by β-cells dysfunction. In the following sections, I will focus on mechanisms implicated in the control of β-cell mass.

Glucose Toxicity

Chronically elevated glucose levels result in glucotoxicity, which contributes to a progressive decline of β-cell function and induction of β-cell apoptosis via multiple mechanisms, such as modulation of proapoptotic to antiapoptotic genes balance: Bcl family members towards apoptosis, thus favouring β-cell death (181), and production of reactive oxygen species (ROS) by hyperactive mitochondria (181-183). ROS is further involved in suppressing insulin response and induction of autophagy impairment (184). β-cells are particularly vulnerable to oxidative stress, possibly due to relatively low levels of antioxidative enzymes (181-183). Furthermore, prolonged, elevated intracellular Ca^{2+} levels are toxic to the β-cells (185, 186). Finally, chronic hyperglycaemia leads to constitutive insulin secretion, which causes ER stress due to the high demand for protein synthesis and processing.

Lipotoxicity

Obesity can be a substantial trigger of T2D by causing hyperlipidaemia and insulin resistance, increasing the demand for β-cells to produce and secrete more insulin into the bloodstream. Higher demand for insulin secretion can, in turn, activate the unfolded protein response (UPR) in overworked β-cells (184). Saturated free fatty acids (FFA), among them palmitic acid, have a toxic effect on β-cells via the generation of ROS, resulting in β-cells apoptosis (184). It was also reported that the synergistic work of palmitate and high glucose levels activate caspase-3, triggering β-cells apoptosis (187, 188). Palmitate decreases the expression of anti-apoptotic Bcl-2 protein (189). Palmitic acid also induces Ca^{2+}-dependent degradation of carboxypeptidase E, which is the final enzyme required for the cleavage of proinsulin to mature insulin (190). Reduced levels of carboxypeptidase E were also observed in the high-fat diet-fed mice (191). It is tempting to suggest that increased levels of unprocessed insulin will induce the UPR and ER stress. Also, stress derived from lipotoxicity can activate the UPR by inhibiting the sarco-endoplasmic reticulum Ca^{2+} ATPase pump (SERCA), leading to the depletion of ER Ca^{2+} stores and the impairment of ER homeostasis (192).

ER stress

β-cells of the pancreas are the only source of insulin in our body. This high secretory demand will force the β-cells to produce and secrete large amounts of insulin and other hormones, like islet amyloid polypeptide (IAPP), making the β-cells susceptible to secretory pathway stress, especially when demand is increased by insulin resistance. Elevated protein flux through the ER might lead to accumulation of misfolded proteins and activation of unfolded protein response (UPR) (193-195). Activated UPR alters ER Ca^{2+} levels and favours proapoptotic signals, degradation of proinsulin mRNA, and induction of IL-1β release, resulting in macrophages recruitment and local islet inflammation (196). Three main ER-resident molecules, PERK, ATF6, and IRE1, sense the ER overwork and trigger adaptation responses, which, if failed, trigger the β-cells apoptosis (194). The transcription factor CHOP is a major mediator of ER-stress-induced apoptosis, and mice lacking the CHOP gene are resistant to ER-stress-induced β-cells death (193, 197). Elevated ER-stress markers are detected in T2D patients, suggesting that ER stress may contribute to β-cells apoptosis during T2D progression. However, it is unclear if ER stress initiates β-cells death before the onset of T2D.

Islet amyloid polypeptide (IAPP)

Amylin, known as islet amyloid polypeptide (IAPP), is a hormone with strong amyloidogenic properties, co-secreted from pancreatic β-cells together with insulin. IAPP regulates gastric emptying and suppresses the secretion of glucagon from α-cells (198). The expression of IAPP is regulated by glucose metabolism and calcium concentrations and is enhanced by fatty acids (199). Increased insulin demand in individuals with insulin resistance, and hence increased IAPP secretion, may result in the non-efficient conversion of immature pro-IAPP to mature IAPP and its accumulation in the ER (200). Reports showed that pro-IAPP is fibrillogenic (201), and thus may lead to the assembly of toxic amyloid aggregates: oligomers and fibrils (200). Amyloid deposits contribute to β-cells loss due to islets membrane disruption (through the formation of pores in the cell membrane) caused by amyloid fibrils. IAPP oligomers can induce the NLRP3 inflammasome, contributing to IL-1β release in T2D individuals (202). Therefore IAPP can induce the β-cells death directly via membrane disruption or indirectly via NLRP3 inflammasome activation and IL-1β release. Rat and mouse IAPP are not amyloidogenic (200).

Pro-inflammatory cytokines

Pro-inflammatory cytokines secretion and immune cell infiltration are characteristic of type 1 and 2 diabetes. In healthy individuals, hyperglycaemia stimulates macrophages to produce IL-1β leading to potentiation of insulin secretion via binding to the IL1 receptor (IL1-R), which is abundantly expressed on β-cells. Subsequently, insulin stimulates macrophage glucose uptake and further IL-1β production creating a loop for glucose disposal (203). Thus, low concentrations of IL-1β potentiate insulin secretion (203, 204), and β-cells proliferation (205, 206). In T2D patients, increased hyperglycaemia and hyperlipidaemia stimulate the secretion of IL-1β from islets themselves, leading to chronic local inflammation and infiltration of islets by macrophages, promoting its apoptosis (207). Moreover, long-term exposure to IL-1β contributes to β-cells exhaustion and death via apoptosis. Reports showed that blocking the IL-1β signalling with IL1-R antagonist anakinra (208, 209), and IL-1β activity with the specific IL-1β antibody canakinumab (208, 210, 211) improved β-cells function. Literature suggests that pro-apoptotic cytokines, such as IL-1, TNFα and INFγ decrease the expression of SERCA pumps that load calcium into ER, which

impairs Ca^{2+} dependent protein processing and promotes ER-stress leading to β-cells apoptosis (193, 212).

Autophagy

Autophagy is a homeostatic "self-eating" process that is highly conserved in all eukaryotes and during which the cells own cytoplasmic components are routed for degradation within the lysosomes (213).

Up to date, different types of autophagy are distinguished (214). Macroautophagy represents the main process responsible for removing damaged organelles and protein aggregates within the cell (214). The first step of macroautophagy encompasses a formation of an isolation membrane (phagophore) from the ER membrane. However, membranes from different components, like Golgi apparatus, endosomes, or plasma membrane that are in close proximity to the ER, can be used to initiate the phagophore formation. Generation of phagophores can be activated at multiple sites in the cytosol (215, 216), and few studies suggest that ER-associated structures, called omegasomes, may serve as phagophore initiation sites (217, 218). Next, the double-membrane phagophore engulfs the cellular components and forms the cytosolic vesicle, called an autophagosome.

Macroautophagy is unique from other intracellular vesicle-mediated trafficking processes because autophagosomes are forming *de novo*, not from pre-existing organelles by membrane budding (219). Fully assembled autophagosomes transport the cargo to the lysosome. The autophagosome inner membrane and cargo are degraded in the lysosome by exposure to the acidic environment and the lysosome's resident hydrolases. Fusion between the autophagosome and lysosome results in autolysosome (219).

The autophagy process can be divided into initiation/nucleation, expansion, and degradation steps (220). Central roles in the autophagy initiation play: ATG1-homolog, UNC51-like kinase 1 and 2 (ULK-1 or ULK-2) complex, and Bcl-2-interacting myosin-like coiled-coil protein (Beclin-1). First, ULK-1 and ULK-2 and the homolog of ATG17-FIP200 interact with ATG13 to form a stable complex located at the phagophore, regardless of nutrient status (221, 222). The association of this complex with the mammalian target of rapamycin complex 1 (mTORC1) is influenced by nutrient availability. Under nutrient-rich conditions, mTORC1 associates with the ULK-ATG13-FIP200 complex and phosphorylates ULK-1/2, inactivating them. Inactivation of mTORC1 by starvation or rapamycin leads to its

dissociation from ULK-1/2, resulting in dephosphorylation of ULK-1/2. Liberated ULK-1/2 phosphorylates ATG13 and FIP200 lading to induction of macroautophagy. ULK-1/2 also phosphorylates Beclin-1 interacting protein: activating molecule in Beclin-1 regulated autophagy (Ambra 1), resulting in the relocation of Beclin-1 to the ER (214). Next, Beclin-1 creates a complex with Vps34, Vps15, and ATG14L. Vsp34, a class III phosphatidylinositol 3 kinase (PI3K), produces phosphatidylinositol 3 phosphate (PI3P), leading to the recruitment of double FYVE-containing protein (DFCP1), PI3P-binding effector protein (WIPI2), and multiple ATG proteins, starting the formation of isolation membrane and phagophore elongation (214).

The second step is the expansion of the phagophore. This step consists of two ubiquitin-like pathways (UBL). These pathways result in the formation of the ATG12-ATG5-ATG16L1 multimeric complex and a lipidated form of microtubule-associated protein 1 light chain 3 (LC3) (214, 223).

In the first pathway, the conjugation of ATG12 to ATG5 occurs thanks to ATG7 (homologous to the E1 ubiquitin-activating enzyme), which activates ATG12, and ATG3 and ATG10 (homologous to the E2 ubiquitin-conjugating enzyme) that allows for ATG5 conjugation to ATG12. Next, ATG16L1 binds to this complex. Interaction between ATG12-ATG5-ATG16L1 stimulates the lipidation of LC3 (214, 223).

In the second pathway, LC3 is firstly cleaved by protease: ATG4 leaving a glycine residue at the C-terminus. Next, LC3 is conjugated to ATG3 via the action of ATG7 (E1-like enzyme). Then, LC3-ATG3 is recruited to the isolation membrane by interacting with ATG3 and ATG12 from the ATG12-ATG5-ATG16L1 complex. The C-terminus of LC3 is conjugated to the polar head of phosphatidylethanolamine (PE)- a major component of membranes, to produce the membrane-associated, lipidated form called LC3-II (214, 223).

LC3-II is specifically and stably associated with autophagosomes and is localized on the outer and inner sides of the autophagosome membrane. Unconjugated LC3 is cytosolic, and it is the major form of LC3 present under normal conditions. Lipidation of LC3 facilitates autophagosome maturation. When the autophagosome is formed, the ATG12-ATG5-ATG16L1 complex leaves the autophagosome. Additionally, the LC3-II from the outer side of the membrane is released into the cytosol (is cleaved by ATG4 protease) and recycled (223).

The last step of the autophagy process includes the fusion of the autophagosome with the lysosome, where lysosomal enzymes hydrolyze the cargo. Two v-SNARE's: VAMP-3 and VAMP-7 have been shown to control the fusion between autophagosomes and lysosomes (224).

Due to the lysosomal degradation of cargo, autophagy contributes to the regulation of lipids, proteins, and carbohydrates metabolism. Metabolites generated by lysosomal degradation are then released into the cytosol.

Several research groups have suggested that compromised autophagy in pancreatic β-cells serves as one of the components of β-cells malfunction in diabetes, and enhanced autophagy induced by insulin resistance is protective against β-cells dysfunction (225, 226).

It was shown that islets from mice with β-cells knockout of ATG7 protein (which is required for autophagosome formation by conjugation of ATG5 to ATG12 and LC3 lipidation) exhibited increased cell death than autophagy-competent islets when challenged with saturated free fatty acids- ER stress inducer. These results suggest that autophagy is important in managing ER stress (227).

β-cells-specific ATG7 knockout mice develop mild, impaired glucose tolerance but not diabetes. However, when these mice were crossed with obese *ob/ob* mice (obesity induces ER stress), these mice developed severe diabetes, increased apoptosis, and decreased β-cells mass. Moreover, the expression of unfolded protein response (UPR) genes was unchanged in ATG7 knockout- *ob/ob* mice but increased in *ob/ob* mice, suggesting that the demand for UPR response due to obesity is unmet in autophagy-deficient β-cells (227).

Mice expressing human IAPP (murine IAPP is soluble and non-amyloidogenic) in pancreatic β-cells displayed mild glucose intolerance. The same mice developed diabetes when crossed to β-cells specific ATG7 knockout mice (228, 229). Additionally, IAPP oligomers were observed in islets of those mice but not in autophagy-competent controls, suggesting that IAPP oligomers are efficiently cleared by autophagy (228, 229).

β-cells with defective autophagy processes are more susceptible to lipotoxicity and IAPP oligomers induced damage. Long-term exposure of β-cells to these factors is associated with autophagy dysfunction (225, 226). Dysfunctional autophagy is also seen in T2D patients (230, 231). Finally, autophagy-deficient β-cells show abnormal morphology and activity of ER and mitochondria, suggesting that

autophagy is critical for the maintenance of mitochondria and ER function (227, 232, 233).

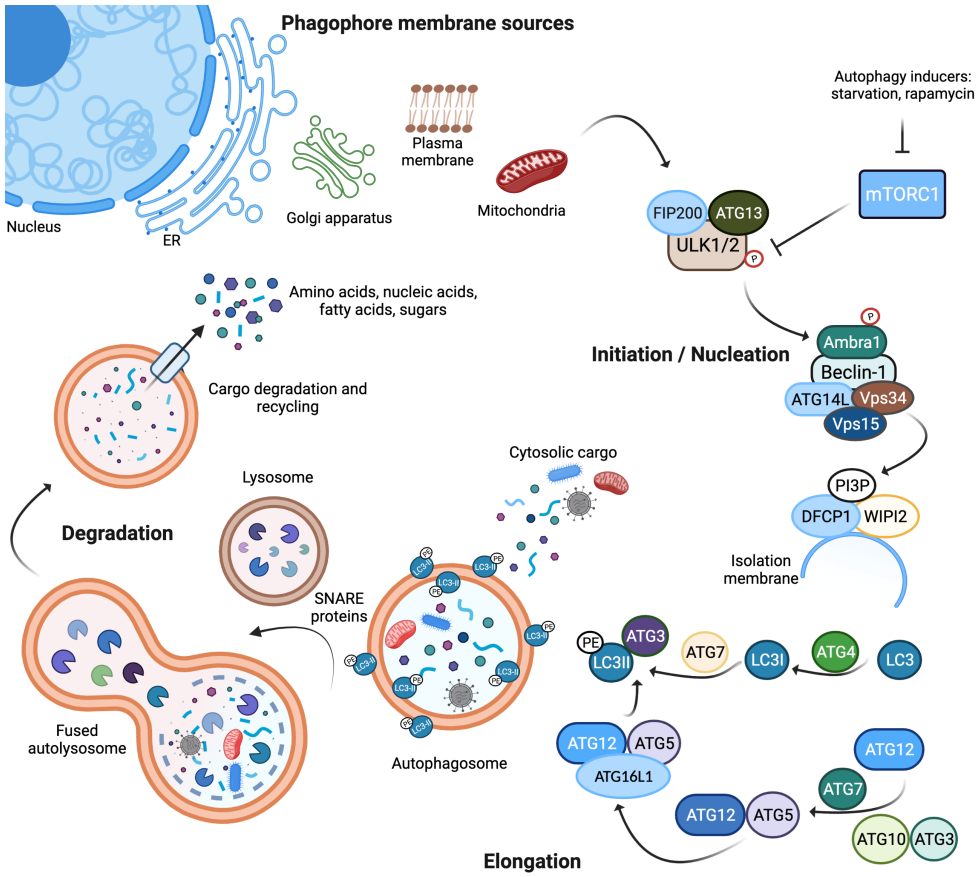

Figure 4. Macroautophagy pathways: Initiation/Nucleation, Elongation and Degradation.

Dedifferentiation

Both β-cells mass and function are affected in T2D. However, there is a debate whether β-cells death or functional defects of β-cells contributes primarily to β-cells failure. It is also unclear whether β-cells mass declines in individuals who progressed to T2D or whether those people had lower β-cells mass at baseline, making them more susceptible to T2D when metabolically challenged.

Traditionally insulin expression was detected by immunolabelling. Using this method, researchers identified β-cells in tissue and quantified β-cells mass. Reduced-insulin positive areas were understood as a loss of β-cells numbers. However, this approach to identifying β-cells may not reflect the actual number of lost β-cells, as loss of insulin staining may occur due to β-cells death, but also due to dedifferentiation and degranulation (234-236).

It was shown that removing half of the human pancreas had minimal effect on diabetes incidence (237). It is tempting to speculate that the functional defects of the β-cells are of higher importance than reduced β-cells mass for causing insulin insufficiency. As described in previous sections, various mechanisms are linked to impaired β-cells function. However, dedifferentiation was recently described as a new and intriguing mechanism for β-cells dysfunction.

Mature β-cells express a set of transcription factors, including *PAX6*, *PDX1*, *NKX 6.1*, *FOXO1*, *NKX 6.2*, and *MAFA* (238-240), and the loss of β-cells transcription factors has been termed dedifferentiation.

Dedifferentiation refers to cells that revert to immature, progenitor-like phenotype. Processes leading to β-cells dedifferentiation are just starting to be elucidated. However, it was shown that β-cells dedifferentiation or loss of identity occurs with prolonged exposure to high glucose conditions (241).

Additionally, it was proposed that the β-cells can also undergo a process called transdifferentiation, which is defined as converting a terminally differentiated cell into another cell type (242).

Literature suggests that islet cells can transdifferentiate into β-cells. Severe β-cells ablation in mice and zebrafish induces α- to β-cells transdifferentiation; but more than 95 % of β-cells must be destroyed to induce this spontaneous transdifferentiation (243, 244). The signal for α- to β-cells transdifferentiation was not identified. However, insulin may act as a repressor of transdifferentiation, and in case of massive β-cells loss, this inhibitory signal is reduced. Huising and

colleagues proposed that transdifferentiation may occur naturally *in vivo* in rodent T2D islets. They identified a subset of insulin-positive cells, which do not express Urocortin 3 (UCN3), or other markers of mature β-cells. Authors termed these cells as "virgin β-cells." Lineage tracing revealed that these virgin β-cells represent an intermediate state between α- to β-cells transition (245).

An interesting study that also points to the plasticity of β-cells was presented by Spijker and colleagues, who using lineage tracing, observed that human β-cells dispersed and re-aggregated *ex vivo* spontaneously converted to produce glucagon (242).

Talachi and colleagues demonstrated that mice lacking β-cells transcription factor *Foxo1* develop diabetes when exposed to physiological stressors, like multiple pregnancies or aging. These mice exhibited a 30 % loss of β-cells number and a 50 % increase in α-cells mass (236). The authors performed a linage tracing in order to determine whether the observed β-cells loss was due to β-cells death or functional defects and found that β-cells loss was due to loss of insulin expression, not due to β-cells death. The authors observed that some cells lost both insulin expression and expression of the β-cells transcription factors: *MAFA* and *PDX1*. Additionally, these cells stained negative for SOX9 (pre-endocrine marker) and expressed high levels of endocrine progenitor markers: NGN3, OCT4, L-MYC, and NANOG, suggesting that these cells dedifferentiated into a progenitor-like state (236). Some former β-cells started to express glucagon, together with *Mafb* transcription factor, which in rodents is generally expressed in α-cells (246). Loss of β-cells and increase in α-cells suggests that β-cells dedifferentiated and started to express other islet hormones, like glucagon. Brereton and colleagues found that in mice with chronic hyperglycemia, 24 % of β-cells expressed neither insulin nor glucagon, and 8 % of β-cells expressed glucagon (247). Additionally, they found that 7 % of the β-cells expressed insulin and glucagon. These bi-hormonal cells retained the expression of PDX1 and GLUT2, but also MAFB, having a phenotype between α - and β-cells (247).

β-cells from *db/db* mouse islets and human T2D donors show loss of mature β-cells transcription factors, like *PDX1* and *MAFA* (235). In the early stages of T2D in humans and *db/db* mice, loss of nuclear *MAFA* is among one of the earliest changes observed in insulin-positive cells (235). Inactivation of *MafA* impairs β-cells function and GSIS without affecting β-cells mass (248).

Another characteristic feature of diabetic mouse models exhibiting dedifferentiation is a mismatched expression of transcription factors.

The expression of glucagon and somatostatin in β-cells was accompanied by the expression of MAFA, PDX1, and NKX 6.1 in diabetic mice (236). In islets from T2D patients, 15 % of glucagon-expressing cells (seven times more than in non-diabetic donors) expressed both *ARX* and *FOXO1*-which is a β-cells transcription factor (249). Additionally, 7.5 % of somatostatin-positive cells expressed *NKX 6.1*. The cellular localization of MAFA and NKX 6.1 in β-cells shifted from nuclear in healthy donors to both nuclear and cytoplasmic in T2D donors (249, 250). White and colleagues published a small case-report study where they found that 1 % of the islets cells from T2D donors expressed both insulin and glucagon whereas no bi-hormonal cells were found in healthy donors (251).

Roles of the Complement system in type 2 diabetes mellitus

During diabetes progression, insulin-dependent cells suffer malnutrition, whereas insulin-independent cells are stressed from a prolonged hyperglycaemic environment. Such condition can generate intracellular (due to accumulation of misfolded proteins) and extracellular (glycated proteins) danger-associated molecular patterns (DAMPs) (252).

The physiological role of the immune response is to remove the DAMPs to maintain homeostasis. However, chronic hyperglycaemic conditions in diabetes lead to chronic inflammation affecting the innate immune system, so normally protective innate immune responses become detrimental in diabetes.

It was reported that diabetes might affect the function of complement proteins and regulators. During diabetes, CD59 can be inactivated by glycation (non-enzymatic reaction). Glycated CD59 is a marker of diabetes, and levels of glycated CD59 in the blood are positively associated with glucose levels (82). Autoantibodies against glycated proteins may initiate the classical pathway complement activation. Additionally, advanced glycation end products (AGEs) induced by hyperglycaemia can serve as neo-epitopes for MBL binding (253). Serum levels of MBL are increased in both T1D and T2D patients (254, 255). We also observe increased MAC deposition in the eyes of patients with diabetic retinopathy, which lead to damage of retinal cells (252).

Increased levels of C3 in plasma are observed in both T1D and T2D patients and are associated with vascular thrombosis (256). Adipocytes are a source of alternative pathway components, such as adipsin, C3, and factor B, leading to the generation of C3a, which will recruit immune cells via C3aR, inducing inflammation and, as a consequence, insulin resistance in adipose tissue. High levels of C3 are present in obese individuals. C3aR KO mice exhibit lower infiltration of macrophages into the adipose tissues resulting in reduced adipose tissue inflammation and improved insulin sensitivity, as adipose tissue is one of the key insulin-sensitive tissues (257).

Adipsin KO mice have also shown reduced inflammation in adipose tissue (258).

Additionally, increased C3 levels are associated with inflammation by enhancing pro-inflammatory cytokines production via C3aR and C5aR signalling (258).

C3adesArg is a C3 cleavage product, which has insulin-like properties and stimulates glucose uptake in adipose tissues (259). C3adesArg enhances adipocyte triglyceride synthesis increasing plasma triglyceride levels, thus participating in increasing insulin resistance (260). Increased plasma levels of C3adesArg are observed in obese individuals and patients with T2D. In obesity and diabetes, the alternative pathway is activated in adipose tissues, creating C3adesArg. C3desArg activates adipose tissue macrophages and accelerates continuous inflammation. Activated macrophages from adipose tissue produce pro-inflammatory cytokines like IL-6 and TNFα, which inhibits the insulin receptor functions and induce insulin resistance. Upon a high-fat diet, C3aR expression is increased in white adipose tissues. Inhibiting C3 can be a potential therapeutic for insulin resistance. Additionally, obesity-associated PAMPs and DAMPs activate the NLRP3 inflammasome in adipose tissues (257, 261, 262).

Methodology

Different materials and methods were used to conduct the experiments presented in this thesis. Here I will discuss the relevance, advantages, and limitations of some of them. The reader can find more detailed methodology descriptions in the papers included in this thesis.

Cell cultures

In most of the experiments presented in this thesis, we used cell lines to model the function of pancreatic β-cells. The usage of cell lines is justified by its many advantages, i.e., cell lines are easier to manipulate genetically and expand for large experiments than primary cells. However, cell line's molecular and functional phenotypes may differ from primary cells. The cell lines used in this thesis are described below.

INS-1 832/13

INS-1 832/13 is a pancreatic β-cell line established from adult rat. This cell line was used for most of the experiments in papers included in this thesis. The advantage of INS-1 832/13 cells is that they are a good model for stimulus-secretion coupling mechanism and that they provide a pure population of insulin-secreting β-cells to study β-cells-specific effects (in contrast to pancreatic islets, consisting of several endocrine cell types). The parental cells of the INS-1 832/13 cell line, the INS-1 insulinoma cells, were created by Asfari and colleagues, who dispersed an x-ray-induced INS1 tumour from NEDH (New England Deaconess Hospital) rats into a culture medium containing β-mercaptoethanol (263). Next, Hohmeier and colleagues developed the INS-1 832/13 sub-clone from the original INS-1 cells by stably transfecting them with the plasmid containing human proinsulin, carrying a geneticin (G418) resistance selection-marker (264).

Therefore plasmids encoding the desired gene and carrying G418 resistance cannot be used to generate stable clones. The INS-1 832/13 cells generated by Hohmeier showed greater insulin secretion in response to glucose than the parental INS-1 cells (8-11 fold compared to 2-4 fold) (264). Additionally, the INS-1 832/13 cells exhibit a glucose response (fold-change of high vs. low glucose) similar to that of primary β-cells. However, these cells depend on the reducing compound: β-mercaptoethanol for growth and phenotype retention.

EndoC-βH1

Many β-cell-lines, commonly in use, have been established from rodents (rats, hamsters, or mice). Therefore, many attempts have been made to generate a human β-cell-line. Ravassard and colleagues transduced the human fetal pancreas with a lentiviral vector expressing oncoprotein SV40LT (Simian Vacuolating Virus 40 Large T Antigen) under the insulin promoter (265). This induced human β-cells proliferation (due to the insulin promoter driving SV40LT expression in β-cells) of fetal pancreatic buds. The transduced pancreases were then grafted under the kidney capsules of SCID mice to develop into pancreatic tissue. Within a few months after transplantation, the SV40LT expressing cells expanded and formed insulinomas. The insulinomas were transduced with a lentiviral vector expressing human telomerase reverse transcriptase (hTERT). The hTERT transduced insulinomas were then grafter into another SCID mice to proliferate the β-cells. After removing the transplanted tissue from SCID mice, cells were dispersed and expanded as cell lines. One of these cell lines: EndoC- βH1, was further characterized. Authors showed that this cell line resembles human β-cells in glucose-stimulated insulin secretion capabilities and is stable for at least 80 passages (265). EndoC- βH1 cell line expresses many specific β-cells markers, has an active cytokine-induced apoptotic pathway, and is responsive to ER stress initiation factors (266). Additionally, transplantation of EndoC- βH1 cells into mice reversed streptozotocin-induced diabetes mellitus (266). EndoC-βH1 cell line is also successfully used as a screening platform to identify new anti-diabetic drugs (266). The disadvantage of this cell line is the slow amplification rate and the smaller insulin content than in native human β-cells.

MIN6

MIN6 is a pancreatic β-cell line established from mice. Miyazaki and colleagues produced transgenic mice harbouring human insulin promoter followed by the SV40LT oncogene, which inactivates tumour suppressor proteins to induce tumour growth in hosts infected with SV40. These transgenic mice developed insulinomas at the age of approximately 13 weeks. Tumours from these transgenic mice were excised and used to create cell lines: MIN6 and MIN7 (267).

MIN6 cells represent a population of insulin-secreting β-cells that are homogenous in morphology and grow in clusters. MIN6 displays the characteristics of pancreatic β-cells, including secretion of insulin in response to secretagogues. However, it has been reported that high passage MIN6 cells (from passage 60 upwards) exhibit impairment or complete loss in their ability to secrete insulin upon glucose stimulation. However, high passage MIN6 cells can still secrete insulin when stimulated with other secretagogues, like potassium (268), which was used to stimulate insulin secretion in paper III.

Primary human islets

Primary human pancreatic islets were used in papers I, III, and IV included in this thesis. The islets are isolated from brain-dead cadaveric multiorgan donors with informed consent obtained from the organ donor or relatives (269). The Nordic Network for Clinical Islet Transplantation (NNCIT) handles the islets from organ donors from Sweden, Norway, and Finland. NNCIT is a collaboration between Nordic transplantation units that transplant the islets to type 1 diabetes (T1D) patient donors as a therapy to improve glycaemic control. All of the islets obtained within the NNCIT are tested for their glucose responsiveness at the islet isolation laboratory at Uppsala University (269). Islets that cannot be used for transplantation are distributed to various research centres in Scandinavia and used for research purposes. In papers included in this thesis, islets cultured for 1-2 days were used, as islets cultured for a longer time gain the inflammatory phenotype.

CRISPR/Cas9 genes editing system

CRISPR/Cas9 system was used in each of the papers included in this thesis to specifically knockout genes: *C3*, *Cd59*, *and PigA* in INS-1 832/13 cell line.

The CRISPR system was first observed in 1987 in E. coli (270). However, the exact functions of these short repeat sequences remained unclear until 2005, when it was shown that these repeated sequences function as a part of an adaptive immune system in Archaea and Bacteria, targeting foreign viral DNA (271). Two distinct RNAs, the CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA), trigger and guide Cas proteins to bind to viral DNA sequences to mediate their cleavage. The tracrRNA is a different type of RNA, interacting with the crRNA to produce the dual guide (g) RNA in CRISPR/Cas systems.

The CRISPR/Cas9 system induces double-stranded breaks (DSBs) in the target DNA, which can be repaired by two DNA repair pathways, homology-directed repair (HDR), and non-homologous end joining (NHEJ) (272). HDR pathway facilitates a DNA repair in the presence of a repair template. In case of the absence of a repair template, DSBs are repaired by the NHEJ pathway, which introduces insertion or deletions into the DNA, resulting in target genes disruption by shifting the reading frame. CRISPR/Cas induced DSBs are repaired predominantly by the efficient eukaryotic cellular NHEJ pathway rather than HDR (273).

Since the first use of the CRISPR/Cas technique as a genome-editing tool in 2013 in mammalian cells (274, 275) this toolbox has been continuously and extensively expanding. CRISPR/Cas system is currently capable of not only manipulating the genomic sequence but also site-specific targeting epigenetic and transcriptional modifications (276).

Although the CRISPR/Cas9 system is a great tool, it has many disadvantages, including relatively low efficiency and the need to screen multiple clones to find the successfully edited cells. Its low efficiency also limits the use of this system in cell lines that proliferate very slowly, like EndoC- βH1. CRISPR/Cas9 technique is also time-consuming. However, the off-target effects are a major concern in CRISPR/Cas9 use in complex eukaryotic organisms. Off-target effects can be defined as unintended cleavages of untargeted genomic sites that display sequence similarity compared to the target site. To minimize the risk of off-target effects, different online platforms have been developed to identify and predict off-target cleavages *in silico*. In each paper presented in this thesis, multiple clones with CRISPR/Cas9 modification have been used. We observed very similar phenotypes between the clones.

Immunostaining and verification of antibodies specificity

Immunostaining was performed in each of the papers included in this thesis on either cell lines or primary human pancreatic islets. The principle behind the immunostaining is the binding between antibodies epitope with antigen expressed in tested cells. The antibody-antigen binding is then visualized with a secondary antibody labelled with a fluorescent tag or peroxidase, which binds to the complex. This method is valuable for verifying the intracellular localization of the protein of interest or its colocalization with other molecules. However, antibodies can often bind unspecifically, resulting in false-positive signals. Therefore, testing antibody specificity is crucial.

The optimal concentrations of used antibodies were assessed by staining of negative control, the INS-1 832/13 CD59 or C3-knockout cells. Staining of these cells with antibodies targeting CD59 or C3 was optimized until no signal for negative control was detected. Additionally, immunostaining using different antibodies generated against the same target was performed to verify if a similar staining pattern is obtained.

Since antibodies against human IRIS-1 and IRIS-2 were homemade (rabbits were vaccinated with peptides derived from the unique C-terminal domains of each IRIS isoform, CQGLKTKQPGKKSAS for CD59-IRIS-1 and CELGYHYVAQAGRRQ for CD59-IRIS-2) detailed characterization of antibodies sensitivity and specificity was conducted.

ELISA assay (with wells coated with recombinant human IRIS-1 and IRIS-2 proteins) was used to assess the sensitivity of generated antibodies. Binding curves presented in paper III verified the high sensitivity of generated antibodies. Additionally, antibodies generated against IRIS-1 did not bind to recombinant IRIS-2 protein, and vice versa.

To assess the antibodies specificity, immunostaining with blocking peptides was conducted. Primary human pancreatic islets and INS-1 832/13 CD59-knockout cells overexpressing human IRIS-1 or IRIS-2 isoform were used for staining (pre-incubation) without or with 1000-fold excess molar concentrations of the peptides used for the generation of the IRIS-1/2 specific antibodies. Blocking the antibodies binding site with peptides resulted in a dramatically reduced signal. The same principle (pre-incubation of the sample without or with blocking peptide) was used for lysates obtained from primary human islets, and cell lines run on Western blot. All the techniques used confirmed the high sensitivity and specificity of generated

antibodies. However, although we can trust the antibodies specificity, additional methods, including proximity-ligation assay (PLA), ELISA, or co-immunoprecipitation, should be used to validate the proteins colocalization.

Present investigations

Complement component C3 is highly expressed in human pancreatic islets and prevents β-cell death via ATG16L1 interaction and autophagy regulation (Paper I)

Hypothesis

The starting point of this study was the finding that C3 is the second (after CD59) most highly expressed complement gene in human pancreatic islets (277) indicating a potential function of C3 in β-cells. Next, the protoarray revealed a binding between C3 and a core autophagy protein, ATG16L1. Therefore we aimed to investigate whether C3 might regulate macroautophagy in β-cells.

Major findings

This study revealed the expected high expression of C3 in human pancreatic islets and its positive correlation with type 2 diabetes donor status, like HbA1c, BMI, and inflammatory markers. Upregulated expression of C3 was found in islets from several rodent diabetes models. C3 was found intracellularly in isolated human pancreatic β-cells. To investigate how C3 may enter the cytosol, we mutated the canonical ATG start site on human C3. We found an alternative ATG start site positioned after the C3 signal peptide that, according to our hypothesis, could be utilized and lead to the production of C3 directly in the cytosol, which was confirmed.

Further, we verified the binding between C3 and ATG16L1 within the cytosol. C3 was found to be required for maintaining autophagy activity in β-cells, as evidenced by the massive accumulation of LC3-II puncta and p62 in pancreatic islets isolated from C3-deficient mice and C3-knockout rat β-cell line (INS-1 832/13), indicating that in the absence of C3 autophagosomes do not fuse with

lysosomes. Autophagy protects the β-cells from injuries caused by exposure to stressors, such as lipotoxicity. When exposing the C3-knockout INS-1 cells to β-cells autophagy inducers (palmitate and IAPP), we observed significantly increased cell death caused by autophagy insufficiency.

Our data suggest that C3 may be upregulated in pancreatic islets during type 2 diabetes as a factor against β-cells dysfunction caused by attenuated autophagy.

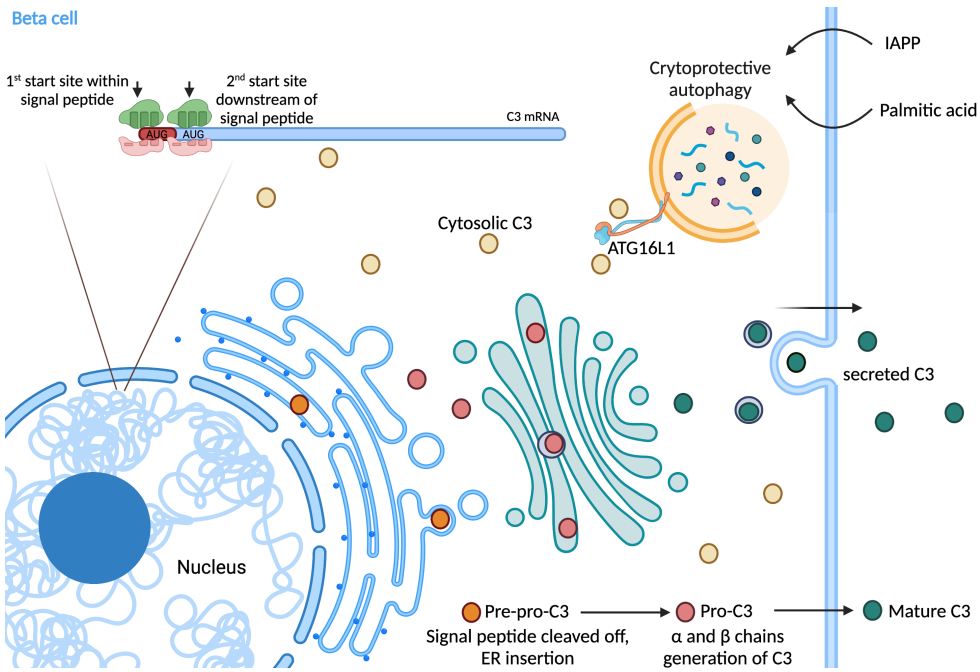

Figure 5. Graphical summary of paper I.

A cryptic non-GPI-anchored cytosolic isoform of CD59 controls insulin exocytosis in pancreatic β-cells by interaction with SNARE proteins (Paper II)

Hypothesis

Previously we revealed a connection between intracellular CD59 and insulin secretion (277) as we found that silencing of CD59 expression in rat β-cells (INS-1 832/13) significantly suppressed insulin secretion. Moreover, removing the membrane-bound CD59 using phospholipase C (which mediates the cleavage of glycosylphosphatidylinositol anchor GPI) did not affect insulin secretion, suggesting that intracellular CD59 is involved in this function. In this paper, we examined various CD59 mutations introduced to the CD59 sequence to investigate which features of CD59 are involved in its intracellular localization and insulin exocytosis to understand how this surface-bound GPI anchored protein can have two such different functions.

Major findings

This study revealed that the CD59 mutant, which lacks the GPI-anchor, was present intracellularly in rat pancreatic β-cell line (INS-1 832/13). We found that non-GPI anchored CD59 was transported from the endoplasmic reticulum (ER) into the cytosol in an N-linked glycosylation-dependent manner, where it interacts with SNARE protein: VAMP2 and rescues insulin secretion in cells lacking endogenous CD59 expression. Therefore we showed that the GPI-anchor, which is necessary for CD59 complement inhibitory function, is not necessary for its ability to mediate insulin secretion. Two other mutations: W40E and C64Y, rescued insulin secretion. Studies showed that these mutations result in a loss of CD59 complement inhibitory functions (79, 100). Following CD59 mutants: N16G-mutant lacking N-linked glycosylation and D24R failed to rescue insulin secretion, suggesting the requirement for the aspartic acid residue at position 24 and glycosylation for the function of intracellular CD59 in insulin secretion. Studies demonstrated that CD59 glycosylation is not essential for its complement regulatory function (not-glycosylated CD59 on the cell surface is still able to inhibit membrane attack complex-MAC). A double mutant lacking the GPI-anchor and N-linked glycosylation: N79STOP-N16G failed to reach the cytosol,

confirming that N-glycosylation is required for retro-translocation of CD59 from the ER to the cytoplasm. Finally, cells with knocked-out PIGA gene (encoding a protein required for GPI anchor synthesis and, therefore, lacking membrane-bound GPI anchored proteins) could still secrete insulin upon glucose stimulation, but not when CD59 was knocked-out, confirming that intracellular CD59 is needed for insulin exocytosis.

Our data suggest that there are different structural requirements for separate functions of CD59, which are: MAC inhibition and insulin secretion.

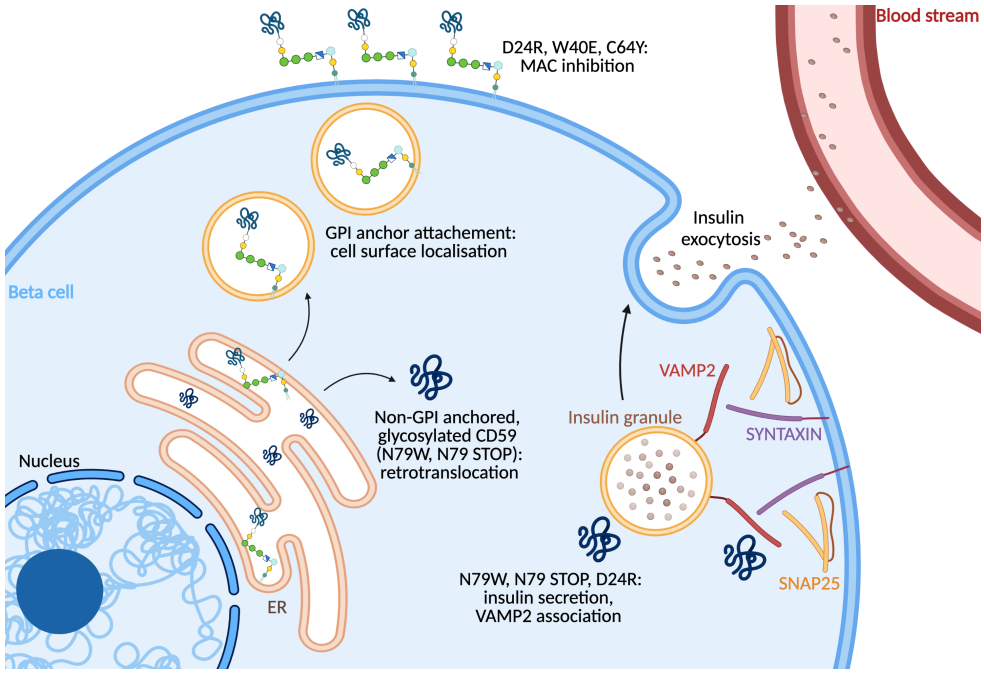

Figure 6. Graphical summary of paper II.

Alternative splicing encodes novel intracellular CD59 isoforms (IRIS-1 and IRIS-2), which mediate insulin secretion and are downregulated in diabetic islets. Additionally, IRIS-1 interacts with DNA in pancreatic islets, suggesting a potential involvement in the regulation of gene transcription (Paper III and IV).

Hypothesis

Paper III (278) showed that the GPI anchor is dispensable for CD59 role in insulin secretion, implying the involvement of intracellular CD59 in this function. However, non-GPI anchored CD59 resulted from experimentally introduced mutation into the CD59 sequence. Therefore, it was not proven that non-GPI anchored CD59 could exist naturally in pancreatic β-cells or how such form is produced.

Major findings

The analysis of available total RNA sequencing data from human pancreatic islets (where we searched for CD59 splice forms lacking the GPI-anchor) revealed the presence of two isoforms lacking the GPI anchoring domain (replaced with the unique C-terminal domains). We named these isoforms IRIS-1 and IRIS-2 (**I**soforms **R**escuing **I**nsulin **S**ecretion **1** and **2**). IRIS-1 lacks the canonical exon 4, which encodes the GPI anchor. The C-terminal domain of IRIS-1 is derived from adjacent predicted open reading frame C11orf91 (uncharacterized so far). IRIS-2, in contrast to canonical CD59, has additionally inserted exon between canonical exons 3 and 4, causing a frame-shift at the C-terminus, resulting in 47 % similarity between IRIS-2 and canonical CD59. We found that both isoforms exist at RNA and protein levels in human and mouse pancreatic islets. They colocalize with insulin granules and interact with SNARE exocytotic machinery VAMP2 and SNAP25, allowing for insulin secretion.

We found that each IRIS isoform permits different phases of insulin secretion. IRIS-1 is involved in releasing a readily releasable pool of insulin granules, which are secreted during the 1st phase of insulin secretion, whereas IRIS-2 is involved in the 2nd phase of insulin release.

Induction of glucotoxicity (by prolonged exposure to high glucose concentrations) in primary, healthy human islets led to a significant decrease in IRIS-1 protein-level expression.

Further, we found that expression of both IRIS-1 and IRIS-2 is markedly reduced in islets isolated from T2D patients compared to healthy controls, suggesting that hyperglycaemia may be one of the factors resulting in reduced IRIS-1 and IRIS-2 expression in T2D individuals.

Interestingly, an electropositive patch was found in the C-terminal region of IRIS-1, suggesting potential interaction with DNA. Since the C-terminal domain of IRIS-1 differs from IRIS-2 and canonical CD59, this domain may mediate additional interactions and convey other functions than IRIS-1 involvement in insulin secretion. We found that IRIS-1 localizes in the nuclei of pancreatic β-cells, where it binds to histones and might regulate the transcription of the genes. We confirmed that the C-terminal domain of IRIS-1 is localizing it to the nucleus. Since robust localization of IRIS-1 in the nucleus is observed only in some nuclei, it can suggest that IRIS-1 is localizing in the nucleus depending on the differentiation state of the cells or in a subset of cells with different functional relevance.

We found that IRIS-1 expressing cells displayed significantly higher expression levels of *Urocortin 3* and *Pdx1* (markers of mature β-cells, which loss marks the beginning of β-cells dedifferentiation), suggesting that IRIS-1 may be required for maintaining the β-cells identity and function. Finally, we believe that the localization of the IRIS-1 in the cytoplasm and nuclei has different functional consequences. Nuclear IRIS-1 does not contradict its role in the cytoplasm, which is the enhancement of insulin secretion.

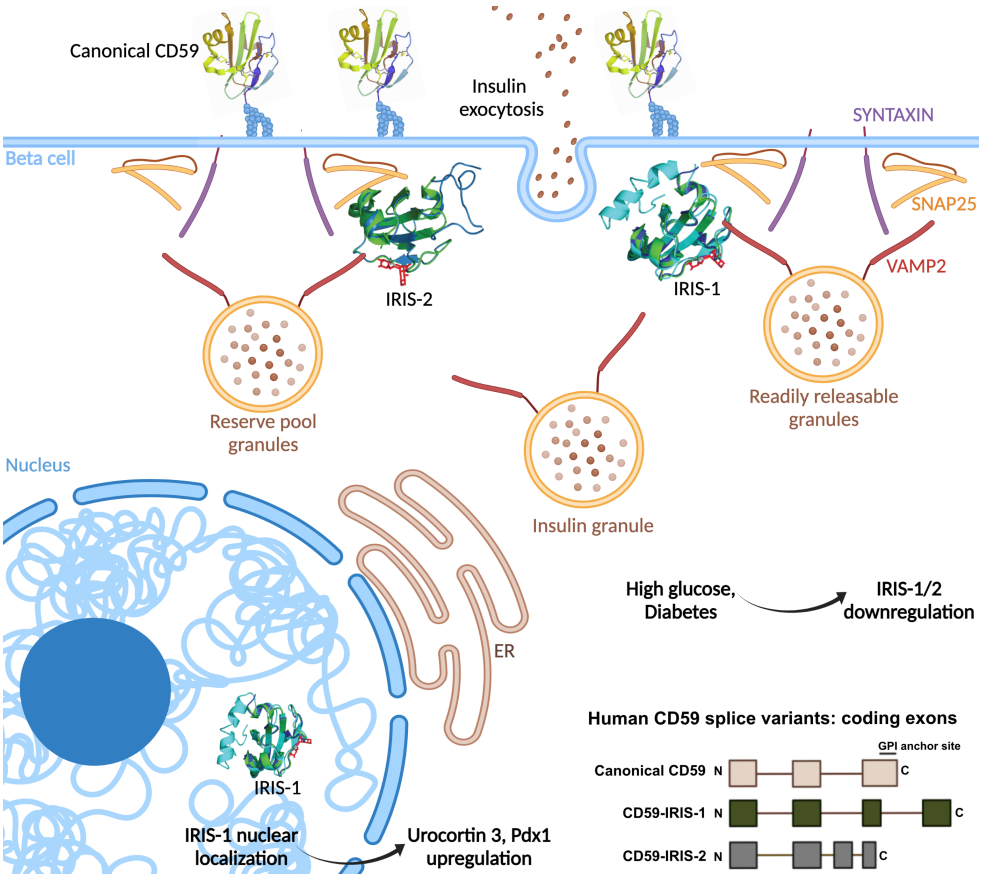

Figure 7. Graphical summary of papers III and IV.

Popular science summary

Diabetes arises when the pancreas does not produce large enough amounts of insulin or when the body cannot use the insulin effectively.

Insulin is a hormone lowering blood sugar levels, as insulin guides sugar transport from the blood into the cells. The cells use sugar as an energy source. Without this action, or when the amounts of secreted insulin are too low, sugar cannot enter the cells and accumulates in the blood. The raised blood sugar level is a main symptom of diabetes, which, if prolonged over time, leads to the development of diabetes complications and premature death.

We distinguish two main types of diabetes: type 1 and type 2.

In type 1 diabetes (T1D) adaptive immune system (which defends the body from pathogens causing infections by producing specific antibodies) mistakenly attacks the body's insulin-producing β-cells of the pancreas, leading to their damage. When the β-cells are damaged, they can no longer produce insulin, and as a result, blood glucose levels rise.

Type 2 diabetes (T2D) is a chronic and progressive disease that drastically increases the risk of developing blindness and heart attack. This type of diabetes affects around 90 % of all diabetic patients. Every year the number of people with diabetes is growing and has reached 537 million people in 2021. Additionally, just in 2021, diabetes contributed to 6.7 million deaths (1 every 5 seconds). In T2D, the body's tissues do not use insulin effectively, which increases the need for higher insulin secretion from the β-cells, leading to their overwork, subsequent exhaustion, and dysfunction.

Innate immunity is highly involved in the pathology of diabetes.

The innate immune system is comprised of immune cells, like macrophages, that control the tissues and defend them from potential danger.

Another element of innate immunity is the complement system, made of multiple proteins in the blood that are "complimenting" antibodies in killing bacteria and

damaged surfaces. Some of the complement system proteins are also present inside the cells, where they are involved in insulin secretion or in supporting the processes crucial for maintaining the cell's health.

CD59 is one of the complement proteins. It is present on the outside of the cells (CD59 attaches to the cell membrane) and plays an important role in protecting host cells against destruction by lysis by the innate immune system. However, CD59 is also present inside the cells in the pancreas' insulin-producing β-cells, where it helps in insulin secretion.

We have also identified novel proteins involved in insulin secretion. These proteins are homologs (different forms) of CD59 protein. We named them IRIS-1 and IRIS-2 (Isoforms Rescuing Insulin Secretion 1 and 2). We found that both IRIS-1 and IRIS-2 are expressed in the insulin-producing β-cells in the pancreas, where they bind to SNARE proteins (insulin secretion machinery proteins). The expression of intracellular IRIS-1 and IRIS-2 is strongly reduced in the β-cells of T2D patients and diabetic mice, as compared to healthy controls, and also by prolonged incubation of healthy human pancreatic islets with high glucose concentrations, suggesting that low levels of IRIS-1 and IRIS-2 can be one of the factors leading to T2D development. IRIS-1 was also found in the nuclei of β-cells, where it can act as a factor changing the expression of different genes and therefore affecting cells' behaviour.

Another insight into β-cells physiology was discovering a novel role for another complement protein called C3. C3 has been found to regulate the process called autophagy (self-eating). Autophagy is a process during which a cell digests and recycles its constituent's parts and turns them into energy. Autophagy also prevents the accumulation of damaged proteins or cell components, which allows the cell to function correctly. When autophagy is uncontrolled, it leads to the development of various diseases, including T2D. We found that C3 is necessary for maintaining the autophagy machinery functioning. Additionally, C3 was found to induce β-cells survival when these cells were exposed to factors like saturated fatty acids, which are excessively present in the blood of T2D patients.

In the future, the precise understanding of the roles of C3, CD59, and IRIS-1/2 proteins in autophagy and insulin secretion may lead to the development of new therapeutics which will help maintain β-cells health and function.

Summary and future perspectives

This thesis aimed to elucidate novel intracellular processes mediated by complement components: C3, CD59, and CD59 isoforms IRIS-1 and IRIS-2.

Although we have described the protective role of intracellular C3 against β-cells stress inducers like IAPP or palmitate, due to the C3 requirement for effective autophagy, the precise mechanism of C3 involvement in autophagy remains to be elucidated.

We hypothesize that intracellular C3 deposits on autophagic targets, like damaged organelles, without the need for factor B or adipsin. In order to assess this, oxidized liposomes (resembling damaged organelles) will be incubated with cytoplasmic lysates supplemented with purified C3. The C3 deposition and subsequent ATG16L1 binding to such liposomes should be seen. According to our hypothesis, C3 is recruited early during the autophagy process, i.e., the nucleation step. We expect to localize C3 to autophagosomes positive for ULK- 1/2, Beclin1 or Vps34, but not autolysosomes positive for lysosomal markers.

Additionally, it is highly possible that ATG16L1 is not the only autophagic binding partner for C3. To search for other intracellular binding partners, tagged C3 could be immunoprecipitated from β-cells lysates challenged with autophagy inducers. The 3D structure of C3 in complex with ATG16L1 or additional ligands should be determined to identify the exact interaction surfaces between C3 and ligands, allowing for precise designing of potential modulators of this binding that could be used as therapeutic. We should also distinguish the roles of intra- and extracellular C3 in order to design the therapeutics optimally, considering the cell membrane permeability of the potential drug.

Since we know that an alternative translational start site on the C3 sequence can be used to produce intracellular C3 involved in autophagy, it is possible that additional translational start sites within the C3 sequence may also be utilized. This may result in the production of different C3 isoforms, which will presumably have different binding partners. The existence of additional C3 isoforms should be verified and their function assessed.

Our study discovered the protective role of cytosolic C3 in β-cells under acute metabolic challenges, like IAPP or palmitic acid. However, the constant metabolic challenge of the β-cells may lead to continuously higher C3 secretion, which might promote local inflammation and switch the signaling to favor detrimental effects of the C3 on the β-cells instead of the protective ones. To study the roles of islets derived C3 under acute and chronic conditions, β-cells with specific C3 knockout will be used. Finally, it was shown that cathepsin inhibition (inhibiting intracellular C3 cleavage) in intestinal epithelial cells prevented intestinal injury (279). This suggests that overactivated intracellular C3 may be harmful to the cell and may also serve as a therapeutic target.

In papers II and III, we have shown that intracellular CD59 and its isoforms: IRIS-1 and IRIS-2, bind to SNARE proteins VAMP2 and SNAP25 to mediate insulin secretion. SNARE proteins may, however, not be the only ligands for CD59 to mediate this function.

We ran a protein-protein interaction microarray using recombinant, glycosylated canonical human CD59 and IRIS-1 isoform (which has a unique additional C-terminal domain) and found interaction with a protein called RPH3AL/ Noc2. Noc2 is a protein associated with insulin granules and Rab proteins, Rab3a and Rab27a (280, 281), and was found to be essential for regulated insulin exocytosis. It was shown that mutations in Rab27a and Rab27a-interacting proteins lead to defects in regulated secretion in pancreatic β-cells due to the failure of secretory granules to dock and fuse with the cell membrane (282). We hypothesize that CD59 is involved in the Rab-Noc2 axis and therefore is required for insulin granule docking and secretion. This will be investigated using interaction studies such as proximity ligation assay, colocalization, or co-immunoprecipitation.

To study *in vivo* relevance of CD59 and IRIS isoforms in insulin secretion, mice lacking both CD59A and CD59B will be used.

We will also perform RNA sequencing on CD59-knockout β-cells expressing human IRIS isoforms to analyze the affected molecular pathways in order to uncover further mechanisms by which CD59 isoforms are involved in the physiology of pancreatic islets. This will be followed with appropriate experiments *in vitro* and *in vivo.*

We will check how different conditions occurring in T2D, like chronic ER stress or IL-1β, affect the expression of CD59 isoforms, to understand which factors

present during diabetes development alter IRIS-1 and IRIS-2 expression and functions.

The motifs within IRIS-1 and IRIS-2, responsible for mediating insulin secretion, should be identified. Therefore, various truncated versions of these proteins will be produced and studied for their ability to rescue insulin secretion in cells lacking endogenous CD59 expression. If these results are successful small IRIS-1/2 peptides may have a potential therapeutic use, busting insulin secretion.

It is also possible that IRIS proteins are involved in regulated secretion in additional cell types, which should be investigated.

In paper IV, we detected an electropositive domain on human IRIS-1, suggesting DNA binding. This domain was not found on canonical human CD59. IRIS-1 is detected in the cytosol and nucleus of β-cells. ChIP sequencing (chromatin immunoprecipitation and sequencing) will be carried out to identify the genomic targets of IRIS-1. Few chosen IRIS-1 targeted genes will be confirmed by luciferase reporter assays and Western blot for protein level determination. We will also verify the binding between IRIS-1 and genomic DNA using an EMSA assay (electrophoretic mobility shift assay). We will test the localization of IRIS-1 (nuclear vs. cytosolic) within the primary human islets exposed to different diabetogenic conditions. This will be done using our highly specific and characterized antibodies and confocal microscopy. We will design mutants in the region of IRIS-1, which we predict is responsible for DNA binding. Site-direct mutagenesis followed by stable transfection of plasmids into CD59 KO cells will be used. These cells will be used to determine the nuclear localization of IRIS-1.

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