Exploring mechanisms of insulin secretion regulators using *C. elegans*

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Cover illustration “SMN-1 expression in the germline” by Agnieszka Podraza-Farhanieh

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"I am among those who think that science has great beauty.
A scientist in his laboratory is not only a technician: he is also a child
placed before natural phenomena which impress him like a fairy tale".
– Maria Skłodowska-Curie

Abstract

Diabetes mellitus is a group of disorders characterized by disrupted glucose homeostasis. Diabetes is one of the most dangerous diseases worldwide since it affects currently more than 500 million people. The pathogenesis of the disease is associated with the insufficient production of insulin and is characterized by increased blood glucose levels. Insulin secretion takes place in pancreatic β-cells in the response to elevated glucose levels and is regulated by various factors. This thesis is aimed to understand the functions of three proteins and characterize their novel roles in the regulation of insulin signaling and secretion. The first study showed the role of ENPL-1 in the positive regulation of insulin secretion. Loss of *enpl-1* resulted in reduced insulin signaling and inhibited insulin secretion. Furthermore, we identified proinsulin as a novel client protein of ENPL-1 and showed that ENPL-1 was required for its maturation. The next study was based on the previous findings showing that ASNA-1 is a positive regulator of insulin secretion. Our study showed that ASNA-1 is present in two redox states, oxidized and reduced and that the multiple functions of ASNA-1 are dependent on its redox states. Our analysis showed, that forcing ASNA-1 into the oxidized state, reduced its function of inserting tail-anchored proteins into the endoplasmic reticulum, without affecting the insulin secretion function. In the next study, we focused on the mutual role of both previously mentioned proteins. We identified the interaction of ASNA-1 and ENPL-1 and showed that proinsulin is required for this interaction to take place. Our study indicated that oxidized ASNA-1 rather than the reduced form was likely interacting with ENPL-1. In the last study, we focused on the role of a third protein, SMN-1, and its impact on the regulation of insulin secretion. Our analysis showed that loss of SMN-1 resulted in neuropeptide secretion defect and caused redistribution of insulin from its original place.
In summary, we characterized the functions of three proteins and indicated their importance in the regulation of insulin secretion processes.

Keywords: *C. elegans*, diabetes, insulin, secretion, ASNA-1, ENPL-1, SMN-1

Sammanfattning på svenska

Diabetes mellitus är en grupp sjukdomar som kännetecknas av störd glukoshomeostas. Diabetes är en av de farligaste sjukdomarna för jordens befolkning eftersom den för närvarande drabbar mer än 500 miljoner människor världen över. Sjukdomen är förknippad med otillräcklig produktion av insulin och kännetecknas av ökade glukosnivåer. Insulinutsöndring sker i bukspottskörtelns β-celler som svar på förhöjda glukosnivåer och regleras av olika faktorer. Denna avhandling syftar till att förstå funktionerna hos tre proteiner och karakterisera deras roller i regleringen av insulinsignalering och utsöndring. Den första studien visade betydelsen av ENPL-1 i positiv reglering av insulinutsöndring. Förlust av *enpl-1* resulterade i minskad insulinsignalering och hämmad insulinutsöndring. Dessutom identifierade vi proinsulin som ett nytt klientprotein för ENPL-1 och visade att ENPL-1 krävdes för dess mognad. Nästa studie baserades på tidigare resultat som visar att ASNA-1 är en positiv regulator av insulinutsöndring. Vår studie visade att ASNA-1 är närvarande i två redoxtillstånd, oxiderat och reducerat, och att de multipla funktionerna hos ASNA-1 är beroende av dess redoxtillstånd. Om ASNA-1 tvingades till det oxiderade tillståndet minskade dess funktion att infoga svansförankrade proteiner i det endoplasmatiska retikulumet, utan att det påverkade insulinutsöndringsfunktionen. I nästa studie fokuserade vi på den ömsesidiga rollen för båda tidigare nämnda proteiner. Vi identifierade interaktionen mellan ASNA-1 och ENPL-1 och visade att proinsulin krävs för att denna interaktion ska äga rum. Vår studie visade att oxiderat ASNA-1 snarare än den reducerade formen sannolikt interagerar med ENPL-1. I den sista studien fokuserade vi på rollen av ett tredje protein, SMN-1, och dess inverkan på regleringen av insulinutsöndringen. Förlust av SMN-1 resulterade i en neuropeptidsekretionsdefekt . Sammanfattningsvis karakteriserade vi funktionerna hos tre proteiner som är av betydelse för reglering av insulinutsöndringen.

Nyckelord: *C. elegans*, diabetes, insulin, utsöndring, ASNA-1, ENPL-1, SMN-1

Streszczenie po polsku

Cukrzyca to grupa zaburzeń metabolicznych charakteryzujących się nieprawidłową regulacją glukozy w organizmie. Choroba ta dotyka ponad 500 milionów ludzi na całym świecie, co czyni ja jedna z najniebezpieczniejszych chorób całej populacji. Powstawanie tej choroby związane jest z niewystarczającą produkcją insuliny przez komórki beta wysp trzustkowych i charakteryzuje się podwyższonym poziomem glukozy we krwi. Wydzielanie insuliny jest regulowane przez różne czynniki, w tym szereg białek. Celem tej pracy doktorskiej było odnalezienie i analiza białek, które mogą mieć potencjalna role w tym procesie. Celem pierwszego projektu była analiza funkcji białka o nazwie ENPL-1. Usunięcie tej proteiny z organizmu, zahamowało całkowicie wydzielanie insuliny. Ponadto pokazaliśmy, że ENPL-1 wiąże się z proinsuliną w komórce i obecność tego białka jest konieczna do powstania aktywnej cząsteczki insuliny. Celem drugiego projektu była analiza funkcji białka o nazwie ASNA-1. Wcześniejsze publikacje pokazały, że obecność tego białka jest konieczna do prawidłowego wydzielania insuliny w organizmie. W tej pracy skupiliśmy się na mechanizmie działania. Odkryliśmy ze ASNA-1 występuje w dwóch aktywnych stanach oksydacyjnych: utlenionym i zredukowanym, oraz że wiele funkcji tego białka zależy od tego, w jakim stanie redoks występuje. Nasza analiza wykazała, że wprowadzenie białka ASNA-1 w stan utleniony spowodowało zahamowanie jego funkcji, ale nie wpłynęło na wydzielanie insuliny. Dzięki temu okryliśmy ze to utleniona, a nie redukcyjna ASNA-1, zajmuje się regulacja insuliny. W kolejnym badaniu skupiliśmy się na wspólnej roli obu wspomnianych wcześniej białek. Pokazaliśmy ze ENPL-1 i utleniona ASNA-1 łączą się ze sobą, oraz że obecność proinsuliny jest wymagana, aby ta interakcja miała miejsce. Celem ostatniego projektu była analiza funkcji trzeciego białka SMN-1. Pokazaliśmy ze utrata tego białka spowodowała zahamowanie wydzielania wielu różnych białek, nie tylko insuliny. Ponadto utrata SMN-1 spowodowała, że insulina była wydzielana z innego miejsca niż oryginalnie. Podsumowując, w tej pracy scharakteryzowaliśmy funkcje trzech różnych białek i wskazaliśmy ich znaczenie w regulacji procesów wydzielania insuliny.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Podraza-Farhanieh A**, Natarajan B, Raj D, Kao G, Naredi P.

ENPL-1, the *Caenorhabditis elegans* homolog of GRP94, promotes insulin secretion via regulation of proinsulin processing and maturation

Development 2020; 147(20) dev190082

II. Raj D*, Billing O*, **Podraza-Farhanieh A***, Kraish B, Hemmingsson O, Kao G, Naredi P.
* Equal contribution

Alternative redox forms of ASNA-1 separate insulin signaling from tail-anchored protein targeting and cisplatin resistance in *C. elegans*

Scientific Reports 2021; 11(1): 8678

III. **Podraza-Farhanieh A**, Raj D, Kao G, Naredi P.

Proinsulin dependent interaction between ENPL-1/GRP94 and ASNA-1 in neurons is required to maintain insulin secretion in *C. elegans*

Manuscript under revision

IV. **Podraza-Farhanieh A**, Kraish B, Raj D, Kao G, Naredi P.

Survival Motor Neuron protein (SMN-1) regulates neuropeptide secretion with Gemin2/SMI-1, independently of its Tudor domain

Manuscript

Table of content

Abbreviation

AID	Auxin-Inducible Degron
ASNA-1	Arsenite-translocating ATPase family
BCA	Bicinchoninic acid assay
BiP	Binding immunoglobulin protein
CBD	Client Binding Domain
CPE	Carboxypeptidase E
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9
CTD	C-Terminal Domain
Daf-C	Constitutive Dauer
DCV	Dense core vesicles
DIC	Differential interference contrast
EGCG	Epigallocatechin gallate
EM	Electron Microscopy
ENPL-1	Endoplasmin 1
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
GARP	Glycoprotein A Repetitions Predominant
GD	Gestational Diabetes
GET	Guided Entry of Tail-anchored proteins
GFP	Green Fluorescent Protein
GRP94	Glucose-Regulated Protein 94
HER2	Human Epidermal growth factor Receptor-2
IGF-I/II	Insulin-Like Growth Factor I / II
IGFR	Insulin Growth Factor Receptor
IIS	Insulin and Insulin-like growth factor Signaling
ILPs	Insulin-Like Peptides
LRP6	Low-density lipoprotein Receptor-Related Protein 6
IRS	Insulin Receptor Substrate
LSM	Laser Scanning Microscope
MAPK	Mitogen-Activated Protein Kinase
MD	Middle Domain

mTOR	Mammalian Target of Rapamycin
NGM	Nematode Growth Media
NTD	N-terminal domain
PC1/2/3	Proprotein Convertase 1/2/3
PI3K	Phosphoinositide 3-Kinase
qPCR	Quantitative Polymerase Chain Reaction
ROS	Reactive Oxidative Species
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
sfGFP	Superfolder GFP
SMA	Spinal Muscular Atrophy
SMN-1	Survival of Motor Neuron 1
SNARE	Soluble N-ethylmaleimide-Sensitive Factor Attachment Proteins REceptor
snRNA	Small nuclear RNA
SRP	Signal Recognition Particle
T1D/T2D	Type 1 Diabetes/ Type 2 Diabetes
TAP	Tail-Anchored Protein
TGF-β	Transforming growth factor beta
TiR 1	Transport inhibitor Response 1
TLR	Toll-Like Receptor
TMD	Transmembrane Domain
TRC40	Transmembrane Domain Recognition Complex 40 kDa
UPR	Unfolded Protein Response
XBP-1	X-box Binding Protein 1

1. Introduction

1.1 Diabetes Mellitus

Diabetes mellitus is a group of chronic, metabolic disorders which have a common denominator – problems with glucose homeostasis. The condition appears when the levels of blood glucose are increased for a prolonged time and the organism is unable to produce enough insulin or use the produced insulin to overcome the increased glucose levels. The disease has been known since ancient times since the first indication of diabetes appeared around 250BC and later in the 17th century the term known as - *diabetes mellitus*, was first mentioned. A better understanding of the disease came in the 19th century when scientists discovered that the pancreas is an essential organ and its removal led to the development of severe diabetes (1). This year marks one hundred years of groundbreaking discovery when two scientists Frederick Banting and Charles Best isolated insulin from the canine pancreatic islets and injected it into a diabetic patient saving his life (2,3). This experiment led to the beginning of diabetes treatment worldwide.

1.1.1. Classification

Diabetes Mellitus is classified into three types: Gestational diabetes (GD), Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D). GD develops only during pregnancy. The disease affects approximately 9% of pregnant people worldwide and when untreated, it might cause serious health complications for unborn children (4). T1D is an autoimmune disease that accounts for 5-10% of the total cases of diabetes. This type of diabetes often develops in children and adolescents; however, it is not restricted only to this age group. In patients suffering from T1D, the immune system attacks and destroys the β-cells in the pancreas, leading to a deficiency in insulin production (5). Currently, the precise etiology of T1D is not clearly understood. The factors that contribute to the pathology of the disease are genetic and environmental factors, such as viral infections, which in combination lead to the autoimmune response that triggers the disease (6,7). T2D is the most common form of diabetes and accounts for more than 90% of all cases of the disease (8). The progression of T2D is caused by increased insulin resistance of the

tissues and deficiency in insulin production by the β cells of the pancreas which leads to hyperglycaemia. Initially, the production of insulin is increased, however, the insulin resistance of the body leads to an increased demand for insulin, which as a consequence leads to the failure of the pancreatic β-cells and depletion of insulin production (Figure 1) (9).

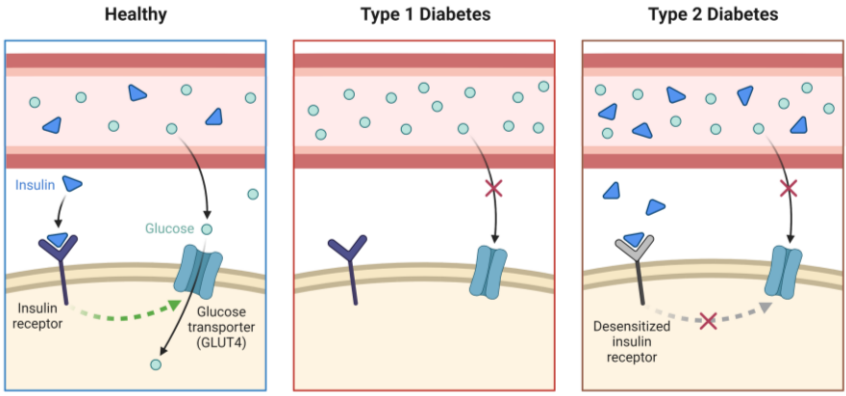

Figure 1. Schematic representation of insulin and glucose relation in healthy, T1D and T2D organisms. *In a healthy organism, insulin in the blood is bound by the insulin receptor on the cell surface. The binding of insulin to insulin receptor allows glucose to be transported by the glucose transporter to the cell. The glucose can be used as an energy source or stored in the form of glycogen in the liver, muscle and fat cells, leading to glucose homeostasis in the organism. In T1D, pancreatic β cells do not produce insulin, which prevents glucose from being transported to the cells, leading to hyperglycaemia. In T2D, increased insulin resistance of the cells leads to the increased production of insulin and, as a consequence, to the failure of insulin production. Desensitized insulin receptor binds the insulin, however, the signaling to the glucose transporter is not properly transmitted, leading to the absence of glucose uptake from the blood (10). Adapted from "Type I vs. Type II Diabetes", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.*

1.1.2. Predictions

Increasing numbers of people who suffer from T1D or develop T2D need effective therapy for disease control and to prevent the unpleasant and deadly consequences of this disease. According to the data reported by International Diabetes Federation, in 2021, 537 million adults worldwide are suffering from diabetes (11). The predictions are not optimistic. It is estimated that within the next decades, diabetes will be the 7th leading cause of death in the world (12). In Sweden, the number of patients suffering from diabetes is relatively low in comparison to other countries in the world, however, the numbers are constantly increasing (13). That is why there is an urgent need for a better understanding of

the biological and biochemical processes of the disease to develop new therapies directed against diabetes.

1.2 *Caenorhabditis elegans*

C. elegans is a species of transparent soil nematode that has been successfully used as a model organism for many years. The pioneer of the *C. elegans* field was Sydney Brenner who studied development and the nervous system of the worm (14). Studies using *C. elegans* significantly contributed to the field of molecular biology and resulted in investigators receiving three Nobel Prize awards for genetic regulation of organ development and apoptosis (15–17), RNA interference (18) and use of GFP in the cell (19).

C. elegans exists in two morphologically distinct forms: hermaphrodite and male (Figure 2a). The hermaphrodite is self-fertile and its genome consists of five pairs of autosomes and two X chromosomes (XX). The male is cross-fertilizing and its genome consists of five pairs of autosomes and one chromosome X (XO). The life cycle of the nematode is relatively short (3.5 days) at 20°C and it is composed of an embryonic stage, four larval stages (L1-L4) and an adult stage (Figure 2b) (20). When the environmental conditions, such as food, temperature and population density are unfavorable for growth, the animal enters a reversible hibernation stage called the dauer stage (21,22).

The genome of *C. elegans* was completely sequenced in 1998 identifying that the vast majority of genes and pathways responsible for the development of human diseases are present in the *C. elegans* (23). It was shown that 60-80% of worm genes have their homologs in protein-coding genes in humans (24). This incredibly high similarity between the human and worm genome, ease of working with this nematode with its short generation time, simple growth requirements, capacity for forward and reverse genetics, as well as a fully mapped cell lineage analysis and neural connectivity, provides a huge opportunity for modelling and studying novel targets of human diseases.

Metabolic dysfunctions such as diabetes and obesity as well as regulators of insulin signaling and secretion have been successfully modelled and studied using *C. elegans* (25–30). The discovery of the DAF-2/insulin receptor in worms (31,32) and the discovery of worm DAF-16/FOXO transcription factor (33–35), provided the foundation to drive the study of insulin signaling in worms and has proven that it is an excellent system to model those biological processes.

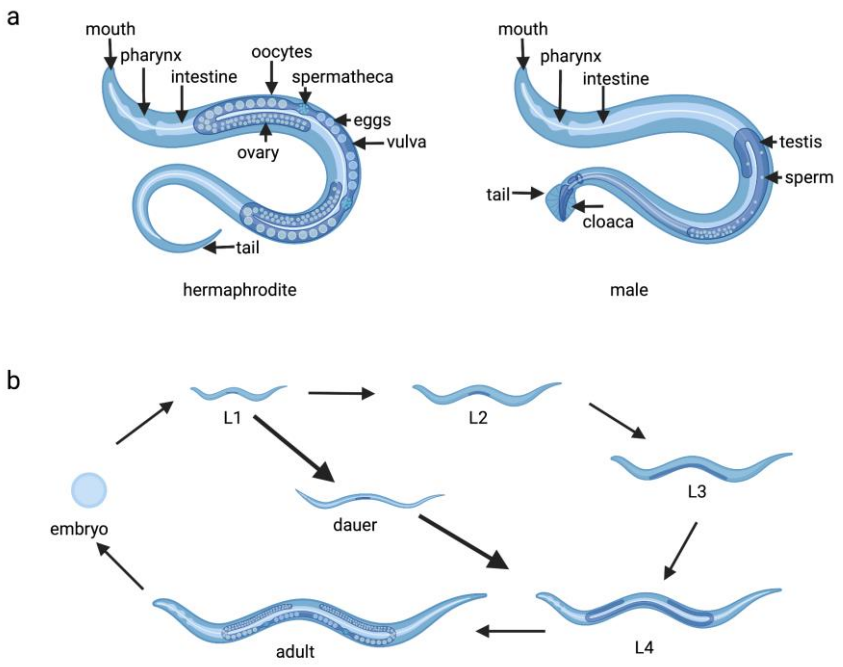

Figure 2. Anatomy and life cycle of C. elegans. *(a) The anatomy of an adult C. elegans hermaphrodite and a male. Arrows indicate the main anatomical features. (b) The life cycle of C. elegans composes of an embryonic stage, four larval stages (L1-L4) and an adult stage. When the growth conditions are not optimal, the animal enters the dauer stage. Created by BioRender.com (2022).*

1.3 Insulin/Insulin-like growth factor signaling pathway

Signaling through insulin and insulin-like growth factors is a widely conserved pathway in vertebrates that associates the availability of nutrients with organismal metabolism, growth, development and longevity. In worms, there is no possibility to distinguish between these two types of ligands, therefore this pathway is called Insulin/Insulin-like growth factor signaling pathway (IIS). Components of the IIS pathway regulate cell survival, proliferation, apoptosis, protein synthesis and glucose metabolism. IIS pathway and its components had been discovered with the help of *C. elegans* genetic analysis of larval development and ageing (27,31,36–39).

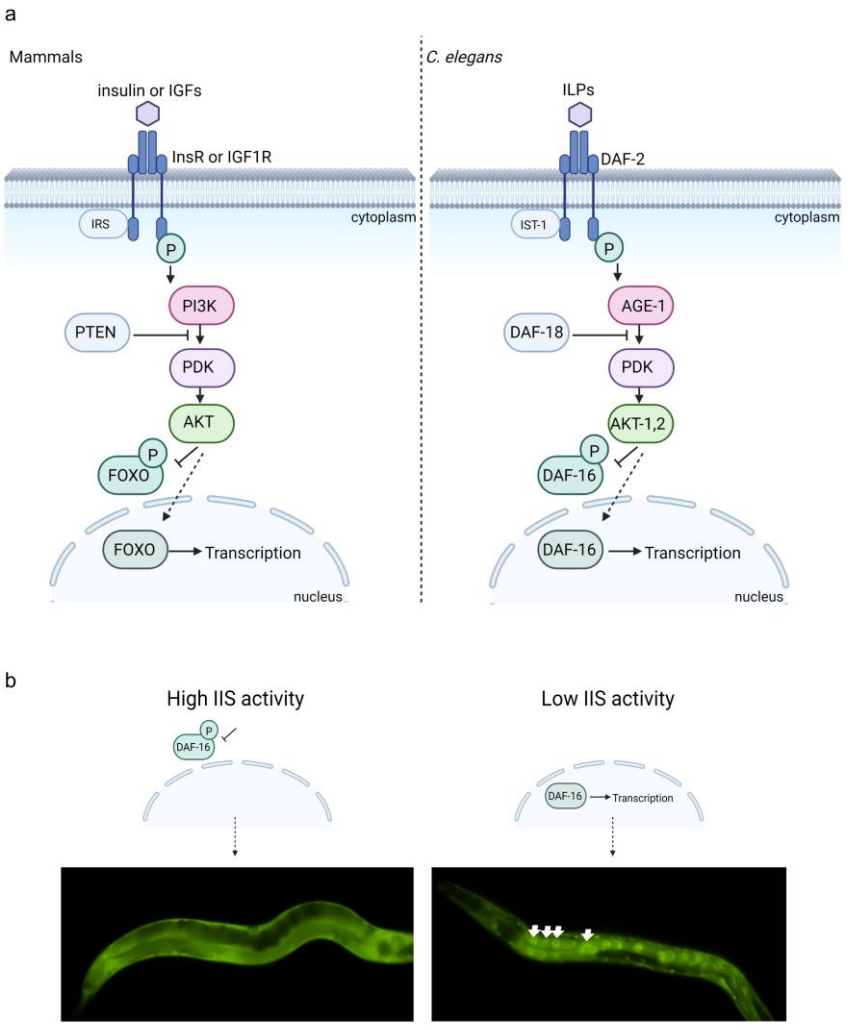

Figure 3. Simplified conserved insulin signaling pathway in mammals and C. elegans*. (a) In the insulin signaling pathway, insulin or IGFs bind to the insulin receptor (InsR)/DAF-2. This leads to the phosphorylation of the insulin receptor substrate (IRS). This event activates PI3K/AGE-1, PDK, AKT-1,2 downstream cascade, which in turn phosphorylates the FOXO/DAF-16 transcription factor. PTEN/DAF-18 activity counteracts PI3K/AGE-1 signaling* (40)*. (b) Representation of high and low IIS activity and the effect on DAF-16::GFP localization. White arrows indicate the nuclei localization of DAF-16::GFP. Created by BioRender.com (2022).*

The IIS pathway is activated by the binding of insulin or insulin-like peptides to the transmembrane receptor called Insulin Receptor (IR)/DAF-2 or Insulin

Growth Factor Receptor (IGFR) (41). Activated receptor phosphorylates insulin receptor substrate (IRS) which promotes the activation of the downstream components of the pathway (42). To the downstream branches of the IIS belong the phosphoinositide 3-kinase (PI3K)/AKT pathway (Figure 3a), Ras/MAPK (mitogen-activated protein kinase) pathway and mTOR (mammalian target of rapamycin) pathway (43). Activation of PI3K/AGE-1 results in activation of PDK and AKT, which as a consequence leads to phosphorylation of FOXO/DAF-16 transcription factor. Phosphorylated FOXO/DAF-16 is prevented from entering the nuclei and remains in the cytoplasm. However, under unfavorable growth conditions, when the IIS activity is low, the non-phosphorylated transcription factor DAF-16 enters the nuclei leading to the activation of gene responses (40,44). The characteristics of nuclear vs cytoplasmic localization of DAF-16 have been widely used in *C. elegans* research to measure the strength of the IIS pathway (Figure 3b) (45).
Additionally, recent findings show that other receptors activate IIS pathways. An alternatively spliced, truncated DAF-2 insulin receptor (DAF-2B) also modifies insulin secretion in *C. elegans* (46) and Insulin/EGF Receptor- L Domain proteins that also modify IIS pathway (47).

1.4 Insulin processing

1.4.1 Insulins synthesis and maturation

Insulin is an essential hormone, produced by the pancreatic β-cells and encoded by the *INS* gene in humans (48). Insulin needs to undergo a series of processing events to create a biologically active hormone (Figure 4).

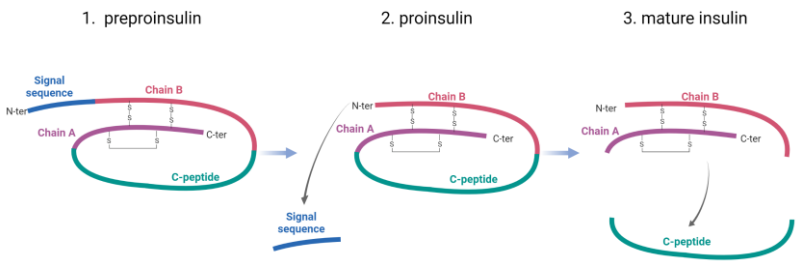

Figure 4. Processing of insulin*. Insulin is synthesized in form of preproinsulin, with N-terminal signal sequence (SS), A-chain, C-peptide and B-chain. The signal peptidase cleaves SS and proinsulin undergoes proper folding, oxidation and disulphide bond formation. Proprotein convertases PC1/3 and PC2 cleave C-peptide, producing mature and biologically active insulin. Adapted from* (49)*. Created by BioRender.com (2022).*

The insulin gene encodes preproinsulin which is the insulin precursor. Because insulin is a secreted peptide, it contains an N-terminal signal sequence peptide that interacts with signal recognition particles (SRP) (50). The SRP allows preproinsulin to be translocated from the ribosomes in the cytoplasm to the ER, where the signal peptidase cleaves signal peptide creating proinsulin. In the ER lumen, proinsulin, which contains A chain, C-peptide and B chain, undergoes proper three-dimensional folding accompanied by the oxidation of cysteine residues to form three disulfide bonds (two inter-chain and one intra-chain disulfide bonds) (51) and creates stable hexamers with zinc ions (51). The folding of proinsulin requires the help of the ER-resident chaperones including GRP94/ENPL-1 (52,53) and PDI (54). After that the proinsulin is transported to the cis- and trans- Golgi (55,56) and enters immature secretory vesicles, called immature dense core vesicles (DCV) (57). Along with proinsulin, to the immature DCV enter processing enzymes. To that class of enzymes belong two proprotein convertases PC1/3 and PC2 and carboxypeptidase E (CPE) (58,59). Maturation of DCV is required for the proper processing of propeptides. Acidification of DCVs activates the enzymes that are required for cleavage of immature proinsulin to mature proinsulin by removal of the C peptide. PC1/3 and PC2 cleave C-peptide and CPE cleaves a few amino acids at the C-terminus of proinsulin (49,60–62). Processed insulin remains in the DCV, while enzymes are packed into constitutive-like vesicles which bud off from the immature DCV. The mature DCVs with insulin cargo are transported along microtubules to the releasing site and upon stimulation, the DCVs are fused with the membrane (63).

1.4.2 Insulin-like peptides in *C. elegans*

The human genome encodes ten insulin-like peptides, including insulin (64), whereas in worms the situation is more complex. The genome of *C. elegans* encodes forty insulin-like peptides (ILPs) and one insulin receptor, DAF-2 (27,29,31,65,66). Most of the ILPs encoding genes are in majority expressed in neurons of *C. elegans* (29,66). Human and worm ILPs share similarities between structure, processing and function. Sequence comparison between human and worm ILPs showed that INS-1 is closely related to human insulin and human insulin can activate worm insulin receptor (65). Another worm ILP, INS-6, forms a structure similar to that of human insulin and can bind and activate human insulin receptor (28). Binding of worm ILPs to the DAF-2 receptor has not been shown so far.

The abundance of insulin-like genes in worms suggests that the functionality of those insulin-like peptides might be redundant and diverse. Loss of *daf-2* insulin receptor leads to constitutive dauer (Daf-c) phenotype (67), however, loss of any of the forty ILPs does not lead to Daf-c dauer formation (66). Interestingly, the only known mutation in one of the insulin-like peptides, which causes severe Daf-c dauer phenotype, similar to *daf-2 (lf)*, was the gain-of-function mutation in *daf-28 (sa191)* (68,69). Additionally, simultaneous loss of multiple insulin-like peptides (INS-4, INS-6 and DAF-28), was sufficient to lead to the Daf-c dauer phenotype (70). Those findings indicate that the insulin-like peptides are interchangeable and their function is redundant.

The differences between worm ILPs led to the creation of different classifications. They have been classified based on the structure of the protein, their effect on the DAF-2 insulin receptor, or enzymes required for processing. One of the first classifications, performed by Pierce et al (65) placed ILPs based on their domain organization into 4 types: Type γ, β, α and multiple chains. Type γ contains 3 canonical disulphide bonds, like human insulin, type β contains also 3 canonical disulphide bonds and additional interchain disulphide bond, type α lacks interchain A6/A11 disulphide bond and multiple chain type that contains insulin with three insulin repeats.

Another type of classification groups ILPs is based on the activation of the DAF-2 insulin receptor, which leads to promotion or inhibition of the dauer formation (65,71–73). Worm ILPs can act as agonists when they likely activate the autophosphorylation of the DAF-2 receptor (all β-type ILPs), or they can act as antagonists when upon the binding to the DAF-2 receptor, the autophosphorylation is likely blocked (70,74). Studies in the field showed that insulin-like genes create networks by regulating each other's expression and creating crosstalk that controls the development and physiology of the animal (75).

The processing of insulin-like peptides is not universal for all the insulins. Human insulin requires 2 proprotein convertases PC1/3 and PC2 for the cleavage of C-peptide which leads to the mature form of insulin (58,59). In worms, there are four PCs: AEX-5, BLI-4 and KPC-1 – homologs of PC1 (76,77) and EGL-3 – a homolog of PC2 (78). It was identified that some of the ILPs which contain RR or KR cleavage sequences are processed by PC2/EGL-3, whereas those ILPs with R-X-X-R site, are cleaved by PC1/KPC-1 enzyme. The processing of the ILPs in worms is dependent on the presence and position of F-peptide and some of the ILPs required both enzymes for cleaving, whereas some do not require any (70).

1.4.3 DAF-28: insulin or insulin-like growth factor?

In mammalian organisms, insulin and insulin-like growth factors are hormones that regulate metabolism, development, growth and differentiation processes. Insulin is secreted by the pancreatic β-cells, whereas IGFs are secreted by the liver and other tissues (79). Insulin and IGF-1 share 50 % sequence homology (80), their receptors are homologous (81) and the ligand binding event leads to activation of the same pathways PI 3-kinase/Akt pathway and Ras/Raf/MAP kinase pathway (82). In spite of the overall similarities, recent findings indicate that insulin and IGF receptors preferentially stimulate different downstream branches of the pathways (83). Furthermore, insulin and IGFs can bind and activate each other's receptors, although with less affinity (84) and IGF-1 is processed similarly to insulin: signal peptide is cleaved and proteases cleave pro-IGF-1 into mature IGF-1 and E-peptide (85).

In *C. elegans* the separation between insulin-like peptides and IGF-like peptides is not fully clear. In worms, there is only one insulin/IGF receptor, DAF-2 (27,31). Based on the structure of the ILPs it is difficult to distinguish between ILPs and IGFs. Although the function of those peptides, the way they are transported and secreted in the cell, or what other proteins affect their secretion might give more understanding for this issue.

During my PhD studies, I was mainly focused on DAF-28 function and secretion. DAF-28 is one of the forty ILPs expressed by the worm (29). DAF-28 acts as an agonist of the DAF-2 receptor (73). DAF-28 is expressed in ASI and ASJ neurons and in the intestines of *C. elegans* (86). However, our recent data indicate that DAF-28 endogenously tagged with mNeonGreen tag is expressed strictly in neurons and no expression is seen in the intestine (Manuscript III). Generally, DAF-28 is considered an insulin-like peptide and that is what it is called, however, there is an alternative nomenclature, calling it IGF (87). The reason why I think it is an ILP, not an IGF is that the secretion of DAF-28 is dependent on the set of proteins required for the DCV processing and exocytosis. Proteins such as *unc-31*, *tom-1, rab-8, rab-3* and *kpc-1* affect the secretion of both DAF-28 in worms and insulin in mammals (53,88). In mammals, IGF-1 is also secreted via Ca^{2+} dependent synaptotagmin exocytosis system (89), however, whether the same type of vesicles is required for secretion of IGFs and insulin, is unknown.

1.5 Insulin secretion

Insulin secretion takes place in pancreatic β-cells in the response to elevated glucose levels. Glucose is taken up in the cell by the glucose transporter/Glut2, is phosphorylated by the glucokinase to glucose-6-phosphate, undergoes glycolysis and enters the TCA cycle, leading to ATP production. Increased ATP levels close the K^+ channels, leading to the depolarization of the membrane. That event leads to the opening of Ca^{2+} channels and the influx of Ca^{2+} ions into the cell. The increased levels of Ca^{2+} ions stimulate DCVs to secrete insulin (90) (Figure 5).

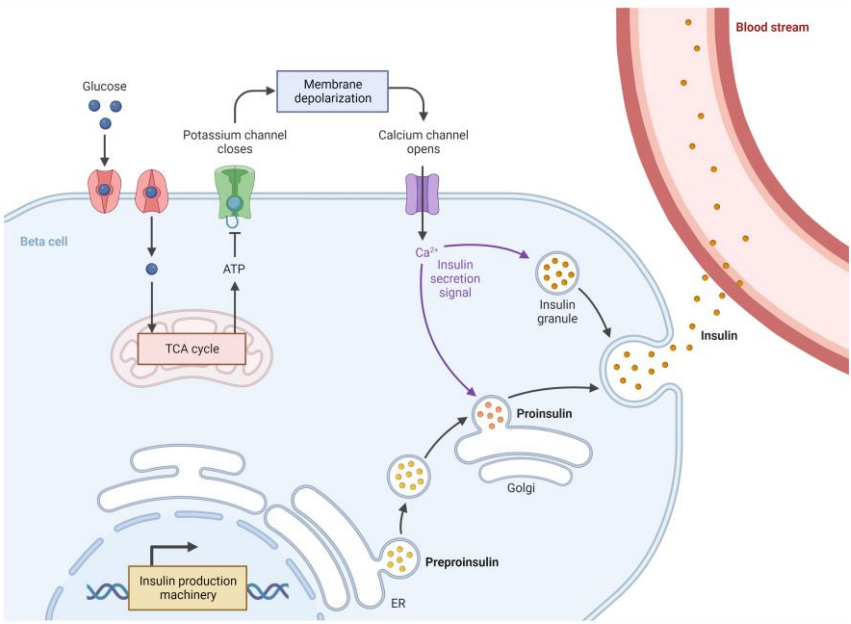

Figure 5. Glucose-stimulated insulin secretion. *Adapted from "Insulin Production Pathway", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.*

The fusion of DCVs with the membrane is necessary for insulin secretion. DCVs dock, prime and fuse with the membrane. Many proteins present on the cell membrane and DCV membrane regulate the fusion processes. During docking, Rab3 interacts with RIM2α (91) and Syntaxin1 binds with Munc-18 which initiates the formation of the docking site (92,93). PI(4,5)P2 lipid has been proposed to have a role in the regulation of syntaxin clustering during docking (94). Priming is required for the proper transformation of the DCVs to the state of

readiness for the fusion with the membrane. During priming the proteins presented on the DCVs and on the membrane create a SNARE (Soluble N-ethylmaleimide-Sensitive Factor Attachment Proteins REceptor) complex (95). T-SNARE (plasma membrane proteins: syntaxin1, SNAP-25) interacts with v-SNARE (DCVs membrane protein: VAMP/ synaptobrevin) and creates a SNARE complex (96–99). Munc-18, munc-13 and CAPS are required for the proper assembly of the SNARE complex (92,100) and tomosyn (101) and complexin (102) stabilize the SNARE complex (103). During Ca^{2+} influx, synaptotagmin regulates exocytosis (104) (Figure 6).

Priming, docking and fusion are relatively conserved processes between mammals and *C. elegans*. Both genomes encode proteins which share similar functions (105–110). In my project, I was working with two components required for the DCV exocytosis: CAPS/UNC-31 and tomosyn. These two proteins have the opposite effects on DCV exocytosis. UNC-31 localizes to DCVs in neuronal cells and regulates Ca^{2+}-dependent exocytosis by proper docking and priming of DCV to the membrane (108,111,112). It was shown that UNC-31 regulates neuropeptide secretion from DCV and does not have a role in secretory vesicle exocytosis (108). Tomosyn is a syntaxin-binding protein and it regulates the secretion of SV and DCV. It was shown that tomosyn inhibits the SNARE complex formation leading to inhibition of DCV or SV priming and secretion (113,114). Loss of *tom-1* function mutants exhibit increased neuropeptide secretion (53,107).

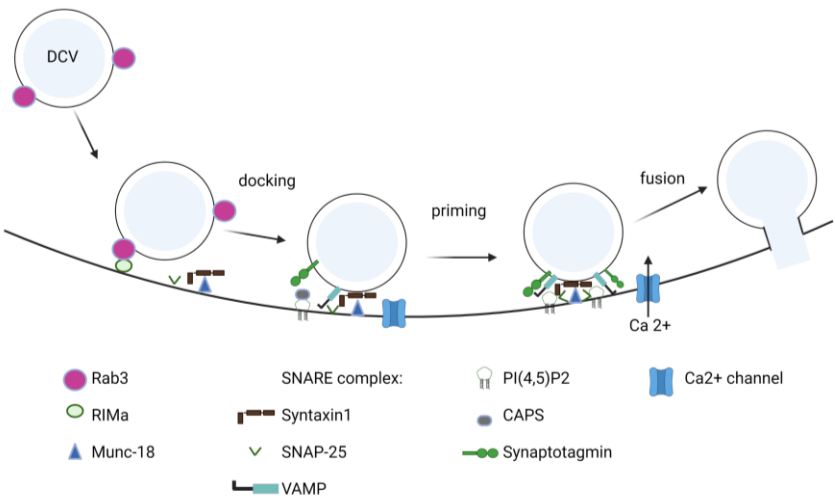

Figure 6. Exocytosis of DCV*. DCV exocytosis is preceded by the docking, priming and fusion of DCV with the membrane. Graphics represents the main components required for the DCV docking, priming and fusion. Adapted from* (103)*. Created by BioRender.com (2022).*

1.6 ASNA-1

ASNA-1/TRC40/Get3 is a conserved ATPase widely expressed in prokaryotes and eukaryotes (115). ASNA-1 was discovered in the bacterial genome as ArsA, a component of the arsenite transport required for the detoxification of metalloids (116). All homologues of ASNA-1 contain a conserved Walker A motif (GKGGVGKT) required for binding of the phosphate group of ATP or GTP and a DTAPTGHT motif with conserved histidines, required for signal transduction (117).

The expression of ASNA1 is essential for organismal growth and development. Loss of *asna1* gene leads to embryonic lethality in mice and a larval arrest phenotype in *C. elegans* (86,118). The subcellular localization of ASNA1 indicates that the protein is mainly localized in the cytoplasm and nucleus (119,120). Additionally, our recent data indicate also a Golgi localization of ASNA-1 (Manuscript III). In mammals, ASNA1 is expressed in several tissues which have a neuroendocrine origin, including the pancreas (119), whereas in *C. elegans* the ASNA-1 is localized in neurons and the intestines (Manuscript III) (86).

1.6.1 Tail-anchored protein insertion

The ASNA1 protein has diverse functions in the cell. The best described and understood role of ASNA-1 is targeting tail-anchored proteins (TAPs) for insertion into the ER membrane (121–123). TA proteins represent 3-5% of all membrane proteins (124) and play roles in many essential functions in the cell including enzymatic reactions, vesicular trafficking, protein localization and regulation of apoptosis (125). The main characteristics of all TA proteins are the presence of an N-terminal cytosolic region, a C-terminal transmembrane domain (TMD) considered as a membrane anchor and a short C-terminal luminal region (126). Importantly, TA proteins lack a signal sequence, hence they cannot be co-translationally inserted into the ER via the SRP mechanism. Their insertion occurs post-transitionally using a highly conserved pathway called Guided Entry of Tail-anchored proteins (GET) described in *Saccharomyces cerevisiae* or called Transmembrane Recognition Complex (TRC) described in mammals in which Get3/TRC40 mediates the insertion (121,123). The simplified model of the insertion of TA proteins into the ER regulated by Get3/ASNA1 is presented in Figure 7. In brief, in the cytoplasm Sgt2 captures synthesized TA protein and presents it to the Get4/5 (127). The Get4/5 complex binds and transfers the TA protein to the ATP-bound Get3 present in a closed state (128). The Get3-TAP complex disassociates from Get4/Get5/Sgt2 due to ATP hydrolysis. Get3-TAP is targeted to the

ER membrane by the Get1/WRB-1 and Get2 - ER transmembrane receptor complex (129). Get3 opens and releases TA protein, which then later interacts with the Get1 and is inserted into the ER membrane. Get3 binds ATP and leaves the membrane ready for the next cycle of TA protein binding (130). Interestingly, it was thought for a long time that all ASNA1 functions are caused by the disrupted TA protein insertion, however, the later studies, including ours (Paper II) showed that ASNA1 has other functions, not related to the TAP insertion.

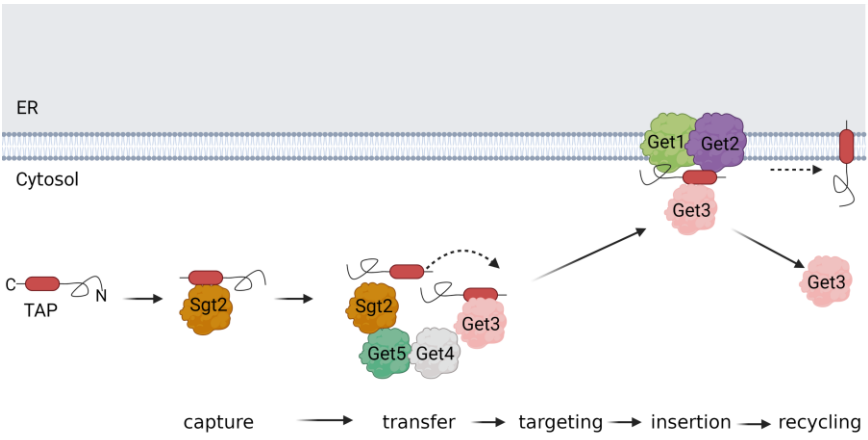

Figure 7. Simplified TA protein insertion by the GET pathway in Saccharomyces cerevisiae. *Adapted from:* (131). *Created by BioRender.com (2022).*

Recent studies have shown that yeast Get3 and *C. elegans* ASNA-1 can be found in two redox-regulated states and it can switch its redox conformation based on the conditions present in the cell (Paper II) (131–133). Under non-stress conditions, Get3 has an ATP-dependent function and is involved in TA protein insertion into the ER membrane. Get3 is present in the dimeric and reduced state and it is stabilized by the zinc coordination of reduced cysteines at positions 285 and 288. TA protein insertion requires ATP binding, necessary for interacting with the TA protein and ATP hydrolysis, that is necessary for releasing Get3 from Get4/5 and for targeting TAP into the membrane (131). However, under the high oxidative stress conditions, Get3 undergoes oxidation changes and switches into an effective ATP-independent chaperone, that protects cells from the damage caused by the high ROS levels (132). The shift from reduced to oxidized Get3 requires structural rearrangements that lead to oxidation of conserved cysteines

and zinc release. This structural change prevents TAP binding to the Get3, however, it creates a chaperone that binds unfolded proteins (132).

1.6.2 Retrograde transport

Besides its role in TA protein insertion, ASNA1 was reported to be required for ensuring proper retrograde transport. It was shown for the first time in yeast that Get3 (at that time still called Arr4) takes part in the transport and retrieval of proteins containing ER-retention sequences from the Golgi to the ER (134). That is why the original name of GET3 meant the Golgi to ER Transport, which later was changed to Guided Entry of Tail-anchored proteins. Later, the studies in mammalian systems further confirmed the findings and showed that the loss of ASNA1 from the pancreas leads to defects in trafficking from the plasma membrane to Golgi and later from Golgi to ER suggesting that the proteins required for ER homeostasis are not retrieved to the ER in the absence of ASNA1 (135). Additionally, loss of ASNA1 from pancreatic β-cells caused Golgi fragmentation (136). The role of ASNA1 in retrograde transport was also correlated with the novel role of ASNA1 in the regulation of apoptosis. Deletion of ASNA1 from the murine pancreas caused defects in pancreas development likely by causing apoptosis of multipotent progenitor cells (136).

1.6.3 Insulin secretion

Another essential role of ASNA-1 is the regulation of insulin secretion. Studies performed in *C. elegans* and in mammalian cells showed that the loss of *asna1* function causes insulin secretion defects (86). Additionally, overexpression and downregulation of ASNA1 protein levels caused reduced and improved insulin secretion of mammalian cells, indicating that ASNA1 has a role in the promotion of those processes (86). In mammals, ASNA1 is expressed in the pancreatic β-cells and co-expresses with insulin, whereas in *C. elegans*, ASNA-1 is localized in the ASI and ASJ neurons and intestinal cells (83). It is still unknown how exactly ASNA1 regulates insulin secretion, it was shown that the protein expression affects the development of the pancreas and pancreatic β-cells (135,136).

1.6.4 Cisplatin response

Another essential role of ASNA1 is the mediation of cisplatin response in the cell. As I mentioned earlier, ASNA1 was discovered first as a bacterial ArsA protein required for the efflux of sodium arsenite and antimonite from the cell (116). Further studies revealed high homology of bacterial ArsA to the mammalian and *C. elegans* ASNA-1 (86,116,137). In bacteria, ArsA binds to the transmembrane protein ArsB and forms the pump required for the detoxification of metalloids. In eukaryotes, there is no homolog of ArsB, however, loss of *asna-1* in *C. elegans* led to increased sensitivity to antimonite and sodium arsenite, indicating that the protein might have a conserved function in metal resistance (137). Further studies in human melanoma and ovarian cancer cell lines confirmed that loss and downregulation of ASNA1 caused increased sensitivity to sodium arsenite and showed that ASNA1 also regulates response to the chemotherapeutic drug cisplatin (138,139). Cisplatin is an effective platinum-based drug widely used in chemotherapy, although development of resistance to the drug causes a major problem with its functionality (140). Further studies in *C. elegans* elucidated the function of ASNA-1 and showed that the conserved histidine His 164 and two conserved cysteines Cys 285 and Cys 288, which are required for proper TA protein targeting (141), were essential for modulation of cisplatin response (Paper II) (142). Interestingly, the mutation in ASNA-1 that caused cisplatin sensitivity and TAP insertion defect, did not affect the insulin secretion function of ASNA-1 (142), providing an indication that ASNA-1 roles can be separated. Our study (Paper II) contributed further to understanding ASNA-1 function, showing that the TA protein disruption is associated with cisplatin detoxification problems, however, it does not cause insulin secretion failure (133).

1.7 ENPL-1

ENPL-1, the worm homolog of GRP94/HSP90B1/gp96/endoplasmin, is a conserved ATPase and ER lumen chaperone required for organismal development and physiology. GRP94 belongs to the family of HSP90-like proteins and its paralogues are known to act as chaperones in the cytoplasm (HSP90α and β) and mitochondria (TRAP1) to fold and protect proteins from stress (143). The structure of GRP94 and other HSP90-like proteins is conserved. They contain the N-terminal domain (NTD) required for the ATP binding and hydrolysis, the Middle Domain (MD) required for the recognition of clients and ATP hydrolysis and the C-Terminal Domain (CTD) required for protein dimerization (144–146). The difference between GRP94 and HSP90β is the presence of KDEL, an ER retention

signal, in GRP94, which is required for binding to the intracellular receptor which brings the Golgi proteins back to the ER (147). The presence of the KDEL motif in GRP94 is essential since its deletion resulted in the secretion of the protein (148).

GRP94 is an active ATPase. The binding of ADP to GRP94 causes GRP94 to adopt an open conformation. This event allows the binding of the client protein to the exposed client-binding pocket of GRP94. The binding of ATP causes GRP94 to close and possibly trap a client protein (149). The closure of GRP94 is relatively slow compared to other HSP90-like chaperones (150). A recent study showed that another ER-resident chaperone, BiP, binds to GRP94 and is required for the acceleration of GRP94 closure required for the trapping of client protein (151). So far only a few chaperones have been identified to interact with GRP94. MZB1 interacts with GRP94 for proper immunoglobulin biosynthesis (152), CNPY3 interacts with GRP94 for folding of toll-like receptors (153), BiP interacts with GRP94 to efficiently trap proIGFs (151) and our recent findings which show the interaction of ENPL-1 with ASNA-1 is potentially required for proper proinsulin biosynthesis (Manuscript III).

The subcellular localization of GRP94 is relatively broad. Mainly the protein localizes in the ER lumen because of its main role in the chaperoning of ER protein and maintaining ER homeostasis (154). ER is a highly oxidative environment, where many proteins designated for membrane insertion and secretion have to be properly folded and assembled (155). Many of those proteins, including insulin, contain disulfide bonds and their cysteines undergo the oxidation required for their proper function. GRP94 also exist as a transmembrane protein in the ER (156) and it was shown that it translocates to the cell surface of normal and cancer cells (157,158) and translocates to the plasma membrane under the ER stress (159). Additionally, subcellular localization of GRP94 indicated the presence of the protein in the Golgi after being Tyr-phosphorylated (160). It has also been found in the cytosolic fraction (53). Thus, good evidence exists for the roles of GRP94 both within and outside the ER lumen.

1.7.1 Role in organismal growth and development

ENPL-1/GRP94 fulfils many functions in the metazoans and it is essential for organismal growth and development (161–166). The protein is ubiquitously expressed in organisms, especially in secretory tissues, including high expression in the pancreas (167). Null mutations of *grp94* and *enpl-1* result in embryonic lethality in mice models and larval growth arrest in *C. elegans* (164,165). Targeted

and tissue-specific depletion of *grp94* using the cre-loxP system in mice caused disrupted development and high ER stress (168,169).

Interestingly, knockdown of GRP94 from mouse pancreatic β-cells caused defects in pancreas development, mass and function, causing decreased β-cells proliferation and increased apoptosis (167). Recent studies showed an essential phenotype of ENPL-1/GRP94 depletion in the development of diabetes. We showed that ENPL-1 binds to proinsulin and loss of *enpl-1* caused inhibited insulin secretion, downregulation of insulin expression and defects in proinsulin maturation (Paper I) (53). Moreover, it was shown that knockdown of GRP94 from pancreatic β-cells contributed to diabetes phenotype and resulted in degradation of proinsulin (52) via the upregulation of proteasome machinery, which is also increased in the pancreatic islets of T2D patients (170).

1.7.2 ER homeostasis

The roles of GRP94 in the ER are essential for maintaining proper homeostasis and quality control (146,171). Under non-ER stress conditions, BiP binds to three ER Unfolded Protein Response (UPR) sensors: ATF6, IRE1, PERK (172,173). When the ER stress increases, misfolded proteins are created and BiP translocates to bind to misfolded proteins. Dissociation of BiP from the ER UPR transmembrane sensors leads to their activation which results in increased ER chaperone expression (174). It was shown that ER UPR pathway activates *grp94* expression (165,175) via ATF6 and XBP1 branches of ER UPR (176).

An additional function of GRP94 in maintaining proper ER homeostasis is its role in ER-Associated Degradation (ERAD). Proteins that have been misfolded in the ER lumen undergo ERAD by the proteasomes. Proper protein folding and detection of misfolded proteins are important steps during the ERAD (177). It was shown that in the absence of GRP94, its client proteins are often subjected to degradation (178–180). Another study showed that GRP94 interacts with lectin OS-9, an ER resident protein that is required for proper detection of misfolded proteins during the ERAD (181). The function of OS-9 is essential since it works as a sensor to distinguish between folded and misfolded proteins. It was also shown that GRP94/OS-9 complex is rather selective and does not target all misfolded proteins for degradation.

1.7.3 Binding to client-proteins

GRP94 is also important for ensuring the proper assembly of its client proteins. The list of client proteins to which GRP94 binds is relatively short compared to other ER chaperones (182). The clients of GRP94 are receptors and secreted proteins (183). Besides that, there are no common features that those proteins are sharing. To the client proteins which bind to GRP94 belong: immunoglobulins (184), toll-like receptors (proteins that have a role in the innate immune system (185,186), integrins (receptors that facilitate cell adhesion) (187), LRP6 (a receptor of the canonical Wnt pathway) (188), IGF-I, II (169,178), insulin (Paper I) (52,53), and GARP (a docking receptor for TGF-β) (180). Interestingly, interaction with other co-chaperones is required for the binding and folding of GRP94 client proteins. CNPY3 interacts with GRP94 and it is required for Toll-like receptor loading into the GRP94 (153), or BiP binds to GRP94/Pro-IGFs complex to accelerate its closure (151). It is still unknown why and how exactly other co-chaperones affect the function of GRP94, although a recent study on GRP94 and BiP interaction showed that BiP enhances GRP94 function and that could be one reason why GRP94 requires its co-chaperones.

1.7.4 Role in Cancer

The role of GRP94 in tumor development is of great interest. Progression of tumor development leads to increased ER stress levels and results in the upregulation of the expression of chaperones (189). GRP94 works as a molecular marker reporting aggressive behavior of many types of cancers since its expression was upregulated in the cancer types with poor clinical outcomes (190). The role of GRP94 in cancer initiation and progression is associated with the client proteins to which GRP94 binds. Loss of GRP94 causes often misfolding of its client proteins preventing their targeting to their proper destinations.

LRP6 is a client protein of GRP94 that works as a receptor in the Wnt signaling pathway required for cell proliferation, differentiation and migration (191). The impaired Wnt signaling pathway is associated with cancer development (192). Loss of GRP94 resulted in the inhibition of LRP6 export from the ER to the cell surface, causing inhibition of the signaling pathway (179).

An additional client protein of GRP94 which has a role in cancer progression is GARP (180). The GARP protein is required for the regulation and localization of TGF-β on the surface of the T cells. The binding of GARP to the TGF-β is required for its proper localization and function. Studies showed that loss of GRP94 activity resulted in inhibition of GARP release from the ER to the cell

surface (180). GRP94 also binds to integrins (185). Integrins are transmembrane receptors which are responsible for signal transduction, which in the case of cancer development, leads to tumor progression (193). It was shown that loss of GRP94 affected the expression of integrins leading to disruption of cell adhesions (194).

Toll-like receptors (TLR) are client proteins of GRP94 (194). TLRs are membrane-spanning receptors that regulate innate immune responses and recognize pathogens (195). Cancer cells also express TLRs, which play a critical role in tumor development and maintain the microenvironment required for tumor progression. It was shown that GRP94 bind TLRs via association with another chaperone CNPY3 and is required for its proper folding (153).

Another GRP94 client protein is Human epidermal growth factor receptor-2 (HER2). HER2 expression level is used as a molecular marker for cancer cell progression and aggressiveness since overexpression of the receptor is associated with malignancy and bad clinical outcomes (196). It was shown that GRP94 binds HER2 and brings it to the plasma membrane and stabilizes it leading to cell proliferation, invasion and metastasis (158). Additionally, it was shown that overexpression of GRP94 enhanced HER2 function by causing the dimerization of the receptor and stimulation of the downstream pathway (197). Interestingly, in the case of HER2, GRP94 leaves the ER and translocates to the plasma membrane. In this case, GRP94 acts via a different mechanism compared to its other client proteins.

1.8 SMN-1

SMN-1 stands for Survival motor neuron protein and it is an orthologue of human SMN1. Mutations in the *smn1* gene in humans result in reduced expression of the protein and the development of spinal muscular atrophy (SMA), a disease characterized by the degeneration of α-motor neurons in the spinal cord that leads to muscle wasting (198). SMA is an autosomal recessive disease and contributes to the highest number of infant mortality caused by a genetic mutation (199). In mammals, there are two *smn* paralogues: *smn1* and *smn2* which share high similarity at the nucleotide level (99%) (200). The development of SMA disease is associated with either a deletion in the *smn1* gene that causes the deletion of exon 7 and/or 8 or point mutations in other exons. Additionally, a mutation leading to the expression of a truncated version of *smn2* lacking exon 7 leads to enhanced degradation of SMN2 protein and also contributes to the development of the SMA disease (198).

SMN1 is ubiquitously expressed in humans. In mammals, SMN1 is present in many tissues including neurons, muscles, kidneys, liver and pancreas (201). In *C. elegans* the expression was detected in all somatic cells and the germline (Manuscript IV). Subcellular localization of SMN1 is in the cytoplasm and nuclear bodies (201,202).

SMN1 exists in an oligomerized form and has many functional domains required for its oligomerization and binding to other proteins (203). Domains of SMN1 include a lysine-rich domain that is required for binding with Gemin2/SMI-1 and with RNA (204), a conserved tudor domain required for interactions with other proteins and assembly of spliceosomes (205), a proline-rich domain (206) and YG box required for self-oligomerization (207). Mutations in all of the domains have been found in SMA patients, however, the most common one is the mutation in the tudor domain which disrupts the SMN complex formation during splicing (208).

1.8.1 Studies of SMN-1 in *C. elegans*

Extensive studies in *C. elegans* have contributed greatly to the knowledge of *smn-1* gene function and SMA disease development. It was shown that SMN-1 function is conserved between human and *C. elegans* orthologues. The null mutant *smn-1(ok355)* resulted in locomotion dysfunction, reduced lifespan and strict larval arrest in the L4 stage (209). A point mutant *smn-1(cb131)* that encodes an amino acid substitution in the highly conserved residue of exon 2, displayed weaker defects and could be used to study milder forms of SMA disease (210). RNAi knockdown of *smn-1* specifically from neurons caused apoptosis and neuronal death and this defect was successfully rescued by transgenic expression of human SMN1 (211). Additionally, loss of *smn-1* caused impaired endocytosis causing a significant decrease in docked vesicles (212), caused mitochondrial defects by affecting the mitochondrial protein localization (213) as well as expression and splicing of other mitochondrial genes (214). Studies also showed that SMN-1 interacts with SMI-1/Gemin2 for the assembly of spliceosomes (215) and interacts with Plastin 3 to prevent neuronal degeneration (211,216). Recent findings showed that loss of *smn-1* function led to downregulation of *lin-45* expression, showing the contribution of the B-Raf/LIN-45 mediated signaling pathway in the development of SMA disease (217).

1.8.2 Assembly of the small nuclear ribonucleoproteins (snRNPs) and pre-mRNA splicing

The most studied role of SMN1 is its function in the assembly of snRNPs of spliceosomes required for mRNA splicing (218). snRNPs are complexes built of RNA and proteins. snRNPs interact with pre-mRNA and build a spliceosome required for the removal of introns and splicing of pre-mRNA (219). SMN1 functions in a complex with Gemin 2-8 and Unrip, binds to snRNA and chaperones the biogenesis and assembly of snRNPs and its trafficking from the cytoplasm to the nucleus (220) (Figure 8). Loss of SMN1 causes defects in the splicing of multiple genes (214,221,222). It is still unknown why the SMA disease mainly affects the motor neuron function even though the assembly of snRNPs and splicing take place in all cells. This also brings the question of whether the role of SMN1 in the splicing processes is the sole cause of SMA pathogenesis.

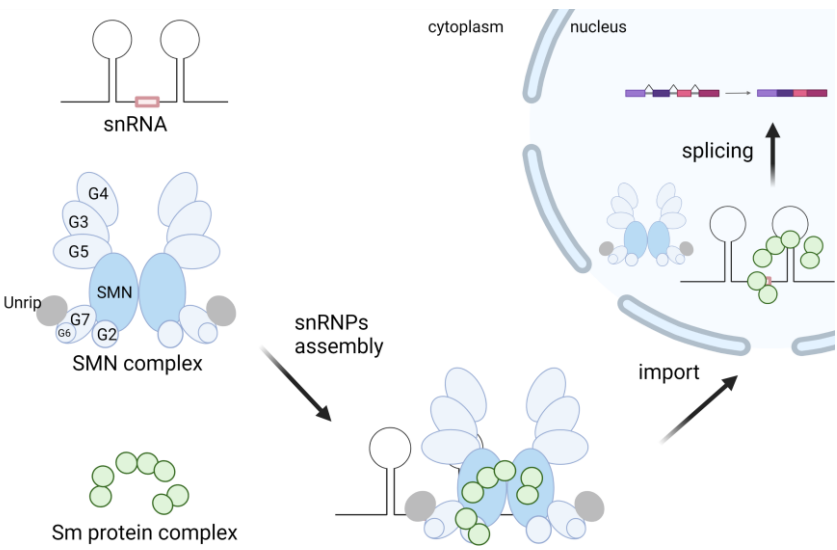

Figure 8. Simplified snRNP assembly pathway. *In the cytoplasm snRNA bind to SMN complex (SMN1, Gemin 2-8, Unirip) and Sm complex (SmB/B', SmD1, SmD2, SmD3, SmE, SmF and SmG). snRNP is assembled and imported to the nucleus. Adapted from* (223) *Created by BioRender.com (2022).*

1.8.3 Role in cell homeostasis

SMN1 protein has multiple roles in maintaining cellular homeostasis. The function of SMN1 has been associated with neuronal growth and development. It was shown that the localization of SMN1 protein shifted during the maturation of neurons, from nuclear to cytoplasmic (224). SMN1 was also found to be transported across the axon in the neurons, co-localizing with Gemin2/SMI-1 but not the Sm protein complex (225) which indicates a non-snRNPs role.

An additional role of SMN1 is the regulation of endocytosis. Loss of *smn-1* in *C. elegans* caused the disruption of endocytosis pathways in the synapses (212). The disrupted neuromuscular junction activity, reduced synaptic transmission and reduced number of docked pre-synaptic vesicles were reported as the main phenotypes observed in the loss of function mutants.

SMN1 has also a role in building a cytoskeleton in the cells, assuring proper localization of β-actin in motor neurons (226). Loss of *smn1* caused a defect in neurite extension and axonal motor axon outgrowth and pathfinding (227). Interestingly, the overexpression of only the C-terminal domain of SMN1 was enough to rescue the neurite outgrowth deficit, which indicates that the snRNPs assembly role is not associated with this function of SMN1 since the tudor domain of SMN1 takes part in snRNP assembly.

SMN1 also has roles in mitochondria homeostasis. *In vivo* analysis showed that loss of *smn1* resulted in the reduction of mitochondria number and impaired transport of mitochondria in the axons. SMA patients had reduced mitochondria numbers in muscles (228). Recent findings showed that loss of *C. elegans smn-1* resulted in differential expression of multiple genes, including mitochondria genes and also their differential splicing (214).

1.8.4 Role in glucose metabolism

SMN1 is also associated with a defect in glucose metabolism. Studies showed that loss of SMN1 in a mouse model resulted in defects in pancreatic composition, leading to the increased number of pancreatic α-cells followed by increased glucagon production and decreased number of pancreatic β-cells followed by reduced expression of insulin (229). This imbalance in pancreatic composition led to increased insulin sensitivity and hyperglucagonemia. Interestingly, the pancreatic composition of SMA patients also shows a similar imbalance of α and β. The continuation of this study indicated that the role of SMN1 in glucose metabolism was independent of the canonical pathology of SMA patients since the

mouse models which they used, did not have classical SMA pathology hallmarks. However, when challenged with a high-fat diet, they did exhibit abnormal pancreas composition (230).
SMN1 appears to have several roles not restricted to splicing. Investigation of these various roles can be performed using *C. elegans*.

2. Aims

The main objective of this thesis was to explore the mechanisms of insulin signaling and insulin secretion regulators to obtain a better understanding of the molecular basis of these processes. Identification and further characterization of the candidates that impact insulin processing and secretion could consequently help to design and develop novel diabetes-targeting drugs.

Paper I

Analysis of the ENPL-1 and its role in promoting DAF-28/ILP secretion and evaluation of its function in the processing of DAF-28.

Paper II

Detection of two alternative redox forms of ASNA-1 and investigation of their separate functions in insulin secretion regulation and cisplatin detoxification mechanisms.

Paper III

Establishing the function of ASNA-1 and ENPL-1 interaction and characterization of their mutual roles.

Paper IV

Characterization of SMN-1 as a novel modifier of neuropeptide secretion and exploring the mechanisms of SMN-1 role in the organism.

3. Methods

This is a brief description of the key methods used throughout the papers. More details can be found in the papers and manuscripts.

***C. elegans* maintenance**
All of the genetic strains were cultured under the standard conditions at 20°C on nematode growth media (NGM) unless otherwise stated. The food source used to culture the animals was an *E. coli* strain called OP50.

Protein isolation
For SDS-PAGE:
Animals were lysed using Next Advance Bullet Blender Homogenizer in a buffer containing 10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5%NP40 using 0.2 mm stainless steel beads for 3 min using a setting of 8, at 4°C, followed by centrifugation at 14,000 rpm for 20 min at 4°C. Protein estimation was carried out using the BCA assay. *For reducing SDS-PAGE*: Protein lysates were boiled 10 min with a loading buffer containing β-mercaptoethanol and SDS. *For non-reducing SDS-PAGE:* Protein lysates were boiled 10 min with a loading buffer containing only SDS (no β-mercaptoethanol). Iodoacetamide in a final concentration of 25mM was added to the boiled sample to avoid post-lysis oxidation.
For pull-down proteomic analysis:
Animals were lysed using Next Advance Bullet Blender Homogenizer in a buffer containing 10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5%NP40 using 0.2 mm stainless steel beads for 3 min at 4°C, followed by centrifugation at 14,000 rpm for 20 min at 4°C. Protein estimation was carried out using a BCA assay. Protein lysates were further subjected to pull-down assays.
For global proteomic analysis:
Animals were lysed using Next Advance Bullet Blender Homogenizer in a buffer containing 2% SDS in 50mM TEAB. Protein estimation was carried out using a BCA assay. Protein lysates were further used for global proteomic analysis.

Western blotting
Protein lysates were separated by electrophoresis using Mini-Protean TGX stain-free gradient precast gels (Biorad) or hand-poured 12.5% gels. Proteins were transferred onto PVDF membranes or nitrocellulose membranes. Membranes

were blocked in 5% skim milk and incubated with antibodies overnight at 4°C. Secondary antibodies were used at a dilution of 1:5000. Proteins were detected using a chemiluminescent detecting system.

Co-immunoprecipitation
Animals were lysed as described above. A maximum of 1000µg of total protein was added to anti-Flag or anti-GFP magnetic beads and tumbled end-over-end for 1 h at 4°C. Beads were separated using a magnet and immunoprecipitated proteins were eluted from the beads by boiling with buffer containing SDS and β-mercaptoethanol. Western blot analysis was performed as described above.

Insulin assays
Adult animals carrying indicated transgenes were anaesthetized in 10 mM levamisole, mounted on 2% agarose glass slides and directly imaged. DAF-16::GFP localization was analyzed in indicated mutants by measuring the number of animals with nuclear DAF-16::GFP. DAF-28::GFP, DAF-28::mNeonGreen, ANF::GFP and ss::GFP were analyzed as uptake of neuropeptides by coelomocytes.

Fluorescence microscopy
Animals were anaesthetized in 10 mM levamisole, mounted on 2% agarose pads on glass slides and directly imaged. Nikon Ni-E microscope, equipped with Hamamatsu Orca flash4.0 camera was used for the imaging.

Confocal microscopy
Animals were anaesthetized in 10 mM levamisole, mounted on 2% agarose pads on glass slides and directly imaged. The fluorescence signal was analyzed at 488nm, 561nm and 647nm using a confocal laser scanning microscope (LSM700, Carl Zeiss) equipped with LD C-Apochromat 40x/ 1.1 W Corr objective and (LSM880, Carl Zeiss) equipped with C-Apochromat 40x/1.2 water immersion objective.

Dauer enhancement assays
Analyzed mutant strains were crossed to *daf-7(e1372)*, *daf-2(e1370)* or *daf-16(mgDf50).* The ability to enhance the Daf-c dauer phenotype was scored at 15°C on plates containing abundant food.

Auxin mediated knockdown
Animals were incubated on NGM plates containing water-soluble auxin (Naphthaleneacetic Acid (K-NAA)) at a concentration of 1mM or 2mM. Plates were

prepared by adding auxin into the agar at 56°C in the darkness. Protein knock-down was measured using fluorescence microscopy.

RNA extraction and Quantitative PCR

Animals were grown on NGM plates. Animals were gently washed off the plates, cleaned from the OP50 and 75µl of Nucleozol was added. Animals were lysed by three rounds of the freeze/thaw method and RNA was isolated using Aurum Total RNA Mini Kit (Bio-Rad). qPCR was performed using KAPA SYBR FAST qPCR Kit (Kapa Biosystems), using the comparative Ct method and results were normalized to the chosen housekeeping genes.

4. Results and discussion

Paper I: ENPL-1, the *Caenorhabditis elegans* homolog of GRP94, promotes insulin secretion via regulation of proinsulin processing and maturation

RESULTS

To understand the expression and localization of ENPL-1 we analyzed a CRISPR/Cas9 knockin of super folder GFP into the genomic locus of ENPL-1. Epifluorescence and confocal microscopy, as well as western blot analysis, showed that ENPL-1 was broadly expressed in the whole organism and in all developmental stages. Strong expression was detected in the neurons and the intestine. To determine the subcellular localization, we constructed strain co-expressing ENPL-1::sfGFP with the intestinal-specific ER marker SP12::mCherry and we found that the ENPL-1 was mainly localized to the ER. Subcellular fractionation which was based on differential ultracentrifugation showed that ENPL-1 was present mainly in the membrane fraction, however, approximately 10% of it was also found outside the ER – in the cytosolic fractions. This indicated that ENPL-1 was also present in the non-ER compartments.

Previous research from our group indicated that *enpl-1* could be a potential candidate that regulates insulin signaling in a manner similar to *asna-1* (231). This analysis was based on an RNAi screen for novel candidates for genes which upon RNAi knockdown resemble the phenotype of *asna-1* deletion mutants. We analyzed the *enpl-1(ok1964)* mutants which were sterile, scrawny and had a high ER stress. To understand whether loss of *enpl-1* causes insulin signaling defect we analyzed the subcellular localization of conserved transcription factor DAF-16::GFP. The nuclear localization of DAF-16 indicates activation of the PI3K/AKT lipid kinase pathway, leading to the induction of gene expression required for insulin signaling, growth and longevity (33). In the *enpl-1* mutants, DAF-16::GFP was found in the nuclei of 40% of examined animals, indicating that the *enpl-1* might have a role in insulin signaling. To further supplement this data we performed a dauer enhancement assay to ask whether the loss of *enpl-1* combined with reduced TGFß pathway activity of *daf-7(e1372)* mutants would promote the constitutive dauer formation (33). Our analysis showed that in the

enpl-1(ok1964);daf-7(e1372) double mutant at 15°C, dauer formation was increased. Consistent with the notion that *enpl-1* affects the insulin/IGF pathway, we did not observe the dauer enhancement in *enpl-1(ok1964);daf-16(mgDf50)* worms. This data together with the DAF-16/transcription factor localization indicated that *enpl-1* had a role in the insulin signaling pathway.

We further asked whether insulin secretion is affected by the loss of *enpl-1.* To do that we analyzed the secretion of DAF-28. DAF-28 was tagged with GFP, expressed by neurons and the intestine and secreted to coelomocytes where the GFP fluorescence can be measured reporting the strength of insulin secretion (86). Loss of *enpl-1* led to insulin secretion inhibition since none of DAF-28::GFP was taken up by coelomocytes. To address whether the coelomocytes were functioning properly we analyzed the secretion of ANF::GFP, a non-insulin neuropeptide (108) and we found that the secretion of ANF::GFP was unaffected by the absence of *enpl-1.* When we overexpressed *enpl-1*, using 3xFlag::ENPL-1, DAF-28::GFP secretion was significantly increased indicating that *enpl-1* is sufficient to increase insulin secretion in *C. elegans.*

High ER stress and increased ER UPR lead to insulin secretion defects and as a consequence might contribute to the development of diabetes mellitus (232). We showed that loss of *enpl-1* led to increased ER stress (53,165). We asked whether the increased ER stress is the reason why *enpl-1* mutants have insulin secretion defects. It has been shown that loss of *ire-1*, one of the main branches of ER UPR, led to the accumulation of DAF-28::GFP in the ER of neuronal cell bodies, blocking the exit of insulin to the axons and dendrites and inhibiting its secretion (233). We asked whether the loss of *enpl-1* would lead to a similar accumulation of DAF-28::GFP insulin in the neuronal cell bodies. Our analysis showed that there was no inhibition of DAF-28::GFP transit since it was found in cell bodies, axons and dendrites of the neuronal cells. Furthermore, to reduce the potential impact of high ER stress, we used a constitutively active version of XBP-1, using spliced XBP-1 expressed from a transgene. XBP-1 works downstream of IRE-1 and leads to the expression of ER chaperones to reduce high ER stress in the cell (234). The expression of *xbp-1s* did not improve the insulin secretion defect of *enpl-1* mutants. Taken together we showed that high ER stress was not the main reason for the inhibited insulin secretion since insulin efficiently left the ER in the *enpl-1* mutants.

Having established the importance of *enpl-1* in the regulation of insulin signaling and secretion, we asked whether the transcriptional and translational levels of insulin were affected by the loss of *enpl-1*. Using qPCR, we analyzed the expression of three insulin-like peptide genes: *ins-4*, *ins-6* and *daf-28*. Our analysis

showed that *ins-4* and *ins-6* were normally expressed however, the expression of *daf-28* was significantly reduced in the *enpl-1* mutants.

Furthermore, we asked whether the translational levels of *daf-28* were also affected. To address that we analyzed double-tagged *ollas::daf-28::myc* transgene expressing worms which allowed us to look at the processing of DAF-28. We validated our method by analyzing the processing of proinsulin in the *kpc-1* mutants. KPC-1 is a proprotein convertase that takes part in proinsulin processing by cleaving it at the RXXR site (76). It has been shown before that in the absence of *kpc-1*, DAF-28 is present only in the unprocessed form (70). Using our method, we confirmed that in the *kpc-1* mutants the processing of DAF-28 was disrupted. Interestingly we found that the loss of *enpl-1* also led to the processing defect of pro-DAF-28 since we were able to detect only the unprocessed form. Taken together we found that the loss of *enpl-1* affected the transcription of the *daf-28* gene and that ENPL-1 had a role in insulin maturation.

Having established that loss of *enpl-1* affects the processing of proinsulin, we asked whether ENPL-1 could bind to pro-DAF-28. To address that we first performed confocal microscopy to analyze the localization of both ENPL-1 and DAF-28. We found that ENPL-1::mKate2 and DAF-28::GFP were co-expressed in the same neuronal cells. Furthermore, we performed the co-immunoprecipitation and asked whether 3xFlag::ENPL-1 interacted with OLLAS::DAF-28::MYC. Our analysis showed that the ENPL-1 immunoprecipitated pro-DAF-28, confirming the physical interaction of those two proteins. We asked which residues of ENPL-1 were required for the interaction with proinsulin. To address that we deleted the client binding domain of ENPL-1, a conserved domain crucial for binding its client proteins (194) and we found that ENPL-1(ΔCBD) was not able to pull down the pro-DAF-28 anymore. Taken together we showed that proinsulin/pro-DAF-28 was a new client binding protein of ENPL-1.

We wanted to ask whether the loss of *enpl-1* affected DCVs. To address that we used the DCV marker – IDA-1::GFP which is a DCV specific transmembrane protein (235). We did not observe any changes in IDA-1::GFP levels in *enpl-1* mutants compared to control, indicating that loss of *enpl-1* likely did not affect the formation of DCVs. To increase the release of insulin from DCVs we analyzed *tom-1/tomosyn* mutants. Tomosyn is a negative regulator of insulin secretion and its depletion leads to increased insulin exocytosis (107). Our analysis showed that in the absence of *tom-1*, DAF-28::GFP secretion was significantly increased. Interestingly, the loss of *tom-1* in the *enpl-1* mutant background led to partial bypass of insulin secretion defect, indicating that in *enpl-1* mutants pro-DAF-28 likely reached the DCVs.

DISCUSSION

The purpose of this study was to understand the role of ENPL-1 in insulin signaling and insulin secretion. A previous publication from our group identified *enpl-1* as a new potential regulator of insulin signaling and insulin secretion in *C. elegans*. RNAi-based screening for genes whose inhibition led to an *asna-1*-like knockdown phenotypes was used as a method to identify such genes (231). This study gave us a basis to further characterize its function. We discovered a novel role of ENPL-1 in proinsulin binding and maturation, found a new client protein of ENPL-1 and characterized the domain by which ENPL-1 interacts with proinsulin.

ENPL-1 is a conserved, multifunctional protein and its homologues, especially mammalian homolog – GRP94, had been widely studied. One of the important roles of GRP94 is the binding of IGFs and the regulation of their production (169,178). Those findings gave us a basis to address whether insulin, whose structure is similar to that of the IGFs (236), can also interact with ENPL-1. We chose to study DAF-28, one of forty insulin-like peptides encoded by *C. elegans* because it behaves functionally like insulin and is regulated by the DCV (88,237). Our hypothesis appeared to be correct since we found direct integration of ENPL-1 to proinsulin.

GRP94 is ubiquitously expressed and it mainly localizes to the ER. However, it had been also presented that GRP94 can be found outside the ER, in the Golgi – where is translocated and undergoes Tyrosine phosphorylation (160) or in the plasma membrane where it regulates the trafficking of HER2 in cancer cells (158). Additionally, ENPL-1 contains an ER retention sequence HSEL, which has been shown to take part in the retrieval of the ER-resident proteins from the Golgi to ER (238). Our data also showed that some fraction of the ENPL-1 can be found outside the ER, in the cytosolic fraction. Interestingly, the fact that ENPL-1/GRP94 can be present in many different compartments in the cell makes us wonder whether its role in regulating insulin secretion is restricted to the ER, or does it reach beyond it? In this study we showed that loss of *enpl-1* did not cause the arrest of proinsulin in the ER, indicating that the proinsulin was able to transfer further to the post-ER compartments. Additionally, we found that the increased DCV secretion in *tom-1* mutants was sufficient to bypass the secretion defect of *enpl-1* mutants, indicating that proinsulin reached the DCV. Taken together our results might suggest that ENPL-1 can function in the post-ER-compartments to regulate insulin secretion.

Recently has been shown that GRP94 requires another co-chaperone – BiP to properly maintain its function (151). The ATPase activity of GRP94 was detected to be relatively slow (150) and the function of BiP is to accelerate the closure of

GRP94. Given the fact that GRP94 requires interaction with co-chaperones, we believe that there are more co-chaperones that are relevant to the function of GRP94. Our next manuscript (Manuscript III) addresses further the new co-chaperone of ENPL-1.

Paper II: Alternative redox forms of ASNA-1 separate insulin signaling from tail-anchored protein targeting and cisplatin resistance in *C. elegans*

RESULTS

A previous study of the yeast homolog of ASNA-1, Get3, gave us an indication that ASNA-1 can be present in two redox-sensitive states (132). To validate those findings and further understand the character of ASNA-1, we asked whether worm ASNA-1 was also present in two redox-sensitive states. To address that we performed non-reducing SDS, followed by the western blot analysis, which allowed us to detect oxidized and reduced ASNA-1. We found that wild-type ASNA-1, as well as ASNA-1::GFP, were present in two redox-sensitive states. To determine whether the oxidation of ASNA-1 was happening via two conserved cysteines at positions 285 and 288 (132), we substituted those cysteines with serines. That substitution prevented the oxidation of ASNA-1 indicating that the cysteines 285 and 288 are redox-sensitive sites for ASNA-1 oxidation. To further understand the oxidation mechanism, we asked whether exposure to hydrogen peroxide, a chemical which increases ROS levels (239) would change the oxidative status of ASNA-1. We showed that 30 min of exposure to 5mM H_2O_2 led to an increase in the oxidation of ASNA-1::GFP. This event was reversible since 2h and 4 h recovery, post H_2O_2 exposure restored oxidized ASNA-1 back to pre-exposure levels. The redox balance was also affected in *sod-2* and *mev-1* mutants, both of which have high oxidative stress levels (240–242). To determine whether we can reduce the oxidized levels of ASNA-1, we exposed worms to anti-oxidant epigallocatechin gallate (EGCG) (243), which had been shown to reduce oxidative stress. Our analysis showed that the exposure to 5.7uM EGCG for 48h led to a decrease in levels of oxidized ASNA-1. Taken together, we showed that ASNA-1 is a redox-sensitive protein, that exists in two states and that the oxidation requires two conserved cysteines.

Previously it had been shown before in yeast and mammals that SEC61ß is a protein which requires ASNA1 for its insertion into the ER (121–123). To understand whether worm ASNA-1 is also required to insert SEC-61ß and to establish the model TA protein, we first performed Co-IP MS/MS analysis. We immunoprecipitated ASNA-1::GFP and analyzed the detected proteins by MS/MS. Our analysis showed that SEC-61ß was pulled down together with ASNA-1, indicating that worm ASNA-1 is interacting with SEC-61ß. To further validate the role of ASNA-1 in TAP insertion, we created a transgenic strain co-expressing under the intestinal promoter two proteins: GFP:: SEC-61ß and mCherry-tagged SP12, a

rough ER-specific protein. By performing confocal microscopy, we observed high levels of co-localization between those two tagged proteins. This co-localization required ASNA-1 since in the *asna-1(ok938)* mutants SEC-61ß was no longer properly inserted into the ER, causing the formation of cytoplasmic foci. Additionally, in the absence of *asna-1*, the total level of SEC-61ß protein was reduced, indicating that the protein might have undergone degradation when not inserted into the ER by ASNA-1. To further understand if the role of ASNA-1 in the promotion of insulin secretion is regulated by the insertion of TA protein, we starved animals. Starvation is known to reduce insulin signaling (45) and we observed that the TAP insertion function was not affected in starved animals. The same was true in *unc-31* and *tom-1* mutants which affect the insulin secretion pathway. *unc-31* mutants have lower insulin signaling because of the defect in DCV exocytosis (108), however, loss of *unc-31* did not cause a TAP insertion defect. *tom-1* loss leads to increased insulin secretion since TOM-1 blocks the fusion of DCV with the target membrane (107). However, the loss of *tom-1* that did not affect the SEC-61β TAP insertion. Taken together our analysis determined that SEC-61ß is a model TA protein of ASNA-1 and gave a basis for the separation of two functions of ASNA-1: insulin secretion and TAP insertion.

Insertion of some TA proteins into the ER is facilitated by ASNA1/Get3 with the support of ER receptor- WRB-1/CAML (244). We asked whether the worm ASNA-1 required WRB-1 for proper TAP insertion. Our Co-IP MS/MS analysis showed that WRB-1 was pulled down with ASNA-1, indicating that those two proteins might interact. Furthermore, we measured the levels of WRB-1 in *asna-1* mutants and we found that the expression of WRB-1 was greatly reduced by the loss of *asna-1*. To confirm the shared function of ASNA-1 and WRB-1 we used our previously established TAP insertion model – GFP::SEC-61ß/mCherry::SP12 and we found that in *wrb-1* mutants, SEC-61ß cannot be efficiently inserted into the ER. Given the fact that WRB-1 was required for TAP insertion in a manner similar to ASNA-1, we asked whether the WRB-1 shares another ASNA-1 function – cisplatin detoxification. ASNA-1 has a role in the modulation of cisplatin response and loss of *asna-1* led to cisplatin sensitivity (142). We showed that the same was true for WRB-1 since the loss of *wrb-1* led to a cisplatin sensitivity phenotype. Taken together, we demonstrated here that WRB-1 shares ASNA-1 function in TAP insertion and cisplatin detoxification.

We showed previously that loss of *asna-1* led to L1 arrest and insulin signaling and secretion defects (86). To maintain and work with *asna-1(ok938)* mutants we use a balanced strain that allows for maternal rescue of *asna-1* animals, allowing

them to grow into sick, small and sterile adults. DIC microscopy of *wrb-1* mutants showed substantial differences compared to *asna-1* mutants. Loss of *wrb-1* did not cause growth and germline development defects, indicating that the loss of *asna-1* was much more severe. Furthermore, we wanted to know whether *asna-1* and *wrb-1* mutants share insulin signaling and insulin secretion defects. We analyzed neuronal/cytoplasmic localization of DAF-16::GFP transcription factor upon *wrb-1* RNAi. We did not observe low IIS signaling in animals lacking *wrb-1* since 100% of the animals exhibited cytoplasmic localization of the transcription factor. We also measured the secretion of DAF-28::GFP to coelomocytes and we also did not observe any reduced insulin secretion compared to wild-type mutants. Taken together, our observation showed that ASNA-1 and WRB-1 share TAP insertion and cisplatin detoxification functions, however, the insulin secretion function of ASNA-1 does not require WRB-1.

Knowing that ASNA-1 can be present in two redox-sensitive states: oxidized and reduced, we asked whether altering the balance and forcing the oxidation of ASNA-1 can cause defects in ASNA-1 functions. To address that we obtained mutants in the conserved histidine 164 residue of ASNA-1, previously shown to have a possible role in the TAP insertion function and cisplatin modulation without affecting insulin secretion (141,142). We found that the mutation of His164 did not affect the levels of ASNA-1 protein since the western blot analysis did not show any change in the expression levels. Next, we measured the oxidation of ASNA-1(ΔHis164)::GFP and we found that the deletion of His164 caused the shift in the balance between reduced and oxidized ASNA-1 towards the oxidized form. Additionally, we observed that *asna-1(ΔHis164)* chromosomal mutants had a TAP insertion defect similar to *asna-1* deletion mutants indicating that the His164 is essential for maintaining proper TAP function. The same was true for the cisplatin modulation function since *asna-1(ΔHis164)* mutants had a defect in cisplatin sensitivity as severe as that seen in *asna-1* mutants. Knowing that His164 mutation in ASNA-1 led to the shift towards the oxidized form and caused TAP and cisplatin defects, we asked whether a high ROS level which increases oxidation of ASNA-1 would cause the cisplatin sensitivity. Our analysis showed that *sod-2(gk257)* and *mev-1(kn1)* mutants were significantly sensitive to cisplatin and *sod-2* RNAi led to significant delocalization of TA proteins. Taken together, our analysis showed that forcing the oxidation of ASNA-1 leads to defects in TAP insertion and cisplatin detoxification similar to the complete depletion of ASNA-1

Having shown that ASNA-1 can be present in two different oxidative states, we next asked whether cisplatin can increase ROS levels and lead to oxidation of

ASNA-1. To address that we first measured the ROS production by cisplatin using H2DCFDA fluorescence analysis. This showed that the cisplatin led to increased ROS levels. Furthermore, we measured the expression of genes involved in oxidative stress response after exposure to cisplatin and we found a significant upregulation of those genes. We showed that cisplatin affected the balance between reduced and oxidized ASNA-1, leading to increased oxidation of ASNA-1 at the expense of reduced form. Interestingly, the TAP insertion defect of animals exposed to cisplatin was as severe as that in *asna-1* mutants but no defect in insulin secretion was observed when animals were exposed to cisplatin. Taken together, our analysis showed that cisplatin caused an increase in ROS production, increase in oxidation of ASNA-1 and defect in TAP insertion, but did not cause any insulin secretion defect indicating that pathways involved in ASNA-1 TAP function and cisplatin detoxification might be distinct from those in insulin secretion regulation.

DISCUSSION

Our analysis showed that ASNA-1 was present in two different redox-sensitive states and that those states were responsible for different ASNA-1 functions. Previous findings in the yeast provided a basis to better understand the role of Get3 and its dual functions (132). These studies showed that the reduced form of Get3 was responsible for TA protein insertion, whereas the oxidized form worked as an ATP-independent chaperone which is required for protecting cells from oxidative stress. Those findings opened a new way of thinking since before it was thought that all ASNA1/Get3/TRC40 functions were correlated to TA protein insertion (141). In our study, we continued further to examine this notion and showed that cisplatin affected the balance between oxidized and reduced ASNA-1 disrupting TA protein insertion. However, this did not cause the insulin secretion defect.

In our study, we separated ASNA-1 function based on its redox state. We showed for the first time in the *in vivo* system that exposure of animals to high ROS levels leads to a shift in the balance from the reduced state to oxidized. Primarily this aspect of different states of ASNA-1 was shown only *in vitro*, in isolated proteins (132). In our paper, we addressed also the fact that the shift between oxidized and reduced ASNA-1 was reversed, which was also addressed before in the *in vitro* modes.

Furthermore, another essential argument which showed that the two different states of ASNA-1 had different roles, was the lack of involvement of WRB-1 receptor in the insulin secretion function. WRB-1 receptor is known to interact with ASNA-1 during the insertion of TAPs into the ER (244). However, our analysis showed that *wrb-1* mutants did not have insulin secretion and signaling

defects, compared to *asna-1* mutants, indicating that regulation of insulin secretion by ASNA-1 does not require WRB-1 and hence does not require TAP insertion machinery. On the contrary, a previous study performed in mice with ASNA1 knockdown from pancreatic β cells showed that there was a mislocalization of Stx5, another TA protein, which caused retrograde transport defect and resulted in the development of diabetes (135). Those differences could be explained by the fact that lox/cre mediated knockdown of ASNA1 from the ß-cells did not lead to a full reduction of ASNA1 expression, but rather only reduced ASNA1 levels by 60%. Additionally, the reduction of ASNA1 levels in the described mouse model might have affected many other ASNA1 functions and the delocalization of Stx5 might not have been the reason for the development of diabetes.

The role of oxidized ASNA-1 is still not fully understood. Previous studies have shown that under non-stress conditional Get3 localizes in the cytoplasm with its TA proteins, whereas under high ROS conditions and low energy levels, Get3 changes its sub-cellular localization and localizes more with unfolded proteins playing the role of the holdase (132,245). This analysis showed that oxidized Get3 acts as a chaperone and is required for protecting cells from the consequences of unfolded proteins. Our study showed that when we force ASNA-1 into the oxidized state by exposing the animals to high ROS levels or cisplatin, we prevent efficient TA protein insertion into the ER, without affecting insulin secretion (133). This could indicate also that the TA protein insertion and cisplatin regulation of ASNA-1 could work via similar mechanisms, however, not in the same way as for insulin secretion regulation. In the next manuscript (Manuscript III) we address more the role of oxidized chaperone ASNA-1 in insulin secretion function.

Paper III: Proinsulin dependent interaction between ENPL-1/GRP94 and ASNA-1 in neurons is required to maintain insulin secretion in *C. elegans.*

RESULTS

Knowing that both proteins have a role in the promotion of insulin secretion, we asked whether they physically interact. To address that we performed co-immunoprecipitation followed by western blot analysis from worms co-expressing ASNA-1::GFP and 3xFlag::ENPL-1 expressed under their own promoters. Our analysis showed that the interaction took place *in-vivo* since 3xFlag::ENPL-1 was detected in immune-precipitates produced by anti-GFP beads that pulled down ASNA-1::GFP. In our previous publications, we showed that ASNA-1 and ENPL-1 were expressed in neurons of *C. elegans* (53,86). Additionally, with our previous and current work we showed that DAF-28::GFP was localized to ASI and ASJ neurons (86) and DAF-28 tagged with mNeon::Green by CRISPR/Cas9 was found in only two pairs of neurons. Knowing that ASNA-1, ENPL-1 and DAF-28/insulin were localized in neurons, we asked whether the interaction between them could take place there. To address that we expressed ASNA-1::GFP in neurons and 3xFlag::ENPL-1 under its own promoter and performed co-immunoprecipitation in worms co-expressing these two constructs. Analysis showed that the same amount of 3xFlag::ENPL-1 interacted with ASNA-1::GFP expressed under its own promoter compared to neuronally expressed ASNA-1, indicating that the interaction took place likely in neurons. Confocal analysis of those two proteins showed also co-expression in neurons. Since ASNA-1 and ENPL-1 affect DAF-28 secretion and DAF-28 itself is produced in ASI neurons, we next asked what the consequence of killing ASI neurons would be for the interaction between the two proteins. To address that we co-expressed the two tagged proteins in a strain which causes ablation of ASI neurons (246). Our analysis showed that when ASI neurons are killed, the interaction between ASNA-1 and ENPL-1 was significantly reduced. Taken together we showed that ASNA-1 was an interacting partner of ENPL-1 and that the interaction took place in insulin-expressing neurons.

One of the functions of ASNA-1 is to regulate insulin secretion in *C. elegans* and mammals (86,135,136). In mice, it has been shown that ASNA1 is found in the pancreatic ß-cells and its function was to regulate pancreatic progenitor cell survival and insulin secretion (86,135,136). A previous publication from our lab showed that worm ASNA-1::GFP, expressed from a multi-copy transgene, was expressed in the ASI and ASK neurons (86). We wanted to know whether the

tagged ASNA-1 in a single copy was also expressed in neurons. To address that we inserted the mNeon::Green::AID tag into the genomic locus of *asna-1* and analyzed the expression pattern. We found that ASNA-1::mNeonGreen was present in neurons of *C. elegans*, outside their nuclei. Furthermore, to address the function of neuronal ASNA-1, we analyzed ASNA-1::mNeonGreen with auxin-mediated protein degradation system (AID) which allowed us to knockdown ASNA-1 from the tissue of interest (247). We crossed in a transgene expressing TIR1 only in neurons into the ASNA-1::mNeonGreen::AID background. After the exposure to auxin we were able to deplete ASNA-1 from neurons. This depletion also led to a significant reduction of DAF-28::GFP secretion, indicating that ASNA-1 is required in neurons to regulate insulin secretion in *C. elegans.* Taken together, we showed here the importance of ASNA-1 in neurons for insulin secretion.

Knowing that ASNA-1 and ENPL-1 localize in insulin-expressing neurons and that the neuronal ASNA-1 regulates insulin secretion, we asked whether the loss of DAF-28 would affect the interaction of the two proteins. To address this, we performed co-immunoprecipitation from worms co-expressing ASNA-1::GFP and 3xFlag::ENPL-1 in *daf-28* mutants. Our analysis showed that loss of *daf-28* led to a significant reduction of interaction between ASNA-1 and ENPL-1. We previously showed that ENPL-1 binds to proinsulin (Paper I) (53) which is why we asked whether the high levels of proinsulin would affect the interaction. To address that we used *kpc-1* mutants, which prevent the processing of proinsulin to insulin, leaving only the unprocessed proinsulin (53,70). Our analysis showed that when proinsulin levels are high, in *kpc-1* mutants, the interaction between ASNA-1 and ENPL-1 was significantly increased. Taken together, we showed that the interaction of ASNA-1 and ENPL-1 was dependent on the levels of pro-DAF-28.

In our previous publication (Paper II) we showed that ASNA-1 is present in both oxidized and reduced states and that the oxidation is promoted by high ROS levels (133). A study of ASNA-1 and its homologues showed that the different oxidation states of ASNA-1 are responsible for different functions of the protein (132,133). Knowing that we asked whether the oxidation status of ASNA-1 contributed to the ability to interact with ENPL-1. We used *sod-2* mutants, which have high endogenous ROS levels (242) and high oxidized ASNA-1 levels (133). Interaction of ASNA-1 and ENPL-1 increased in the *sod-2* mutant background, indicating that likely the oxidized ASNA-1 was more prone to bind to ENPL-1. We previously showed that exposure to hydrogen peroxide increases the oxidation of ASNA-1 (133). We exposed animals to 5mM of H_2O_2 for 30 min and

found that the interaction with ENPL-1 also increased. Taken together, our data showed that oxidized ASNA-1 is likely the binding partner of ENPL-1.

Our previous analysis (Paper I) showed that proinsulin interacted with ENPL-1 via the client binding domain (CBD) of ENPL-1 indicating that proinsulin is a client protein (53). We wanted to know whether ASNA-1 interacted similarly with ENPL-1. Our co-immunoprecipitation analysis showed that when the CB domain of ENPL-1 was deleted, the interaction with ASNA-1 was not affected. This indicated that the ASNA-1 is not a client protein of ENPL-1. Furthermore, knowing that the proinsulin can interact with ENPL-1 and GRP94 (52,53), we asked whether ASNA-1 also was able to interact with proinsulin. Our co-immunoprecipitation analysis showed that ASNA-1 did not immunoprecipitate pro-DAF-28, indicating that the ASNA-1 could not interact with proinsulin. Taken together, our analysis showed that the interaction of ENPL-1 with ASNA-1 and proinsulin likely required different domains.

We showed before that ASNA-1 regulates insulin secretion since the loss of ASNA-1 led to inhibition of insulin secretion in *C. elegans* and mammalian cells (86). We wanted to address whether the overexpression of ENPL-1 could improve the insulin secretion defects of *asna-1* mutants. Previously we showed that overexpression of ENPL-1 was sufficient to increase insulin secretion (53). For this purpose, we overexpressed 3xFlag::ENPL-1 in *asna-1* mutants and showed that the insulin could now be secreted in *asna-1* mutants. Taken together this analysis showed that the ENPL-1 can bypass the requirement of ASNA-1 and improve insulin secretion.

To further address the role of ASNA-1, we performed the global proteomic analysis of *asna-1* mutants and compared it to wild type animals. Previously it was shown that the loss of *asna-1* led to the deformation of Golgi structures when examined by electron microscopy (136). We obtained the set of proteins and the pathways which were altered by the loss of ASNA-1. The reactome enrichment analysis identified the most affected pathways which were related to ER and Golgi trafficking, membrane trafficking and vesicle-mediated transport.

To understand whether the loss of *enpl-1* affected the expression and localization of ASNA-1 we performed western blot analysis and looked for ASNA-1 protein levels in *enpl-1* mutants. We did not observe any changes in the total levels of the ASNA-1 protein. Examination of ASNA-1::mNeonGreen subcellular expression showed that it was detected both in a diffuse cytoplasmic pattern and in a

punctate pattern reminiscent of the Golgi. However, when we analyzed the localization of ASNA-1::mNeonGreen in *enpl-1* mutants, we observed a more diffused pattern which was less localized to Golgi like puncta visible in *enpl-1(+)* animals. Taken together, our analysis showed that loss of *enpl-1* led to changes in the localization of ASNA-1 but not the expression level.

DISCUSSION

It was previously shown that mammalian homologues of ASNA-1 and ENPL-1 were essential for the proper development and function of the pancreas and knockdown of those proteins resulted in the development of diabetes in mice (135,136,167). This has motivated us to continue studies in *C. elegans* to identify the possible mutual functions of those two regulators of insulin secretion. The purpose of this study was to understand if two chaperones, known previously for their role in the regulation of insulin secretion in worms and mammals, can interact and if this interaction is required for insulin secretion.

With the auxin-mediated knockdown, we showed that the loss of ASNA-1 specifically from neurons was sufficient to reduce insulin secretion in *C. elegans.* This indicates that the neurons of *C. elegans* which express ASNA-1 and DAF-28/insulin might be considered as a ''pancreas'' of worms. Of course, the comparison is rather broad, however, the neuronal-specific knockdown of ASNA-1 resulted in insulin secretion defects was similar to the murine knockdown of ASNA1 from the pancreas which resulted also in insulin secretion defects and diabetes (136).

For a long time, ASNA1 was considered only a regulator of tail-anchored protein insertion (121). Many thought that the phenotypes caused by loss of ASNA1 or by its downregulation were associated strictly with the impaired insertion of TA proteins into the ER. Our data, together with previous publications, show that the role of ASNA1 is also non-TAP dependent (132,133). The presence of the two forms of ASNA-1 and their independent functions show that the non-TAP-related role is of big interest in the regulation of insulin secretion. Our current results, together with the previously published ones, show that retrograde transport might be the essential reason which regulates insulin secretion processes (135,136). It was shown that loss of ASNA1 from pancreatic β-cells led to impaired development of pancreatic progenitor cells and affected the retrograde transport. Also, the novel localization of ASNA1 in Golgi structures and the defect in Golgi morphology observed in the loss of ASNA1 (136) indicates further its role at the level of Golgi, which is essential for insulin biosynthesis and maturation.

In this study, we show that ENPL-1 and ASNA-1 interact and that the oxidized ASNA-1 likely binds ENPL-1. As was shown before, oxidized ASNA-1 has a non-TAP-related role and it is considered to be a general chaperone to bind other proteins (132,133). Generally, ENPL-1 or its mammalian homolog GRP94 is an important ER chaperone that regulates ER homeostasis by binding and folding insulin, IGFs and toll-like receptors (52,53,178). The clientele of the GRP94 is rather restricted and limited compared with other ER chaperones. The ATPase activity of GRP94, which is essential for binding the client-proteins, is relatively low (150,248). Recent studies indicated that there is a need for a co-chaperone to enhance the binding to those designated clients. BiP is considered a novel binding partner of GRP94, which is required for the acceleration of the closure of GRP94 to capture the IGF-1 (151). Although, whether the same co-chaperone is required for binding of GRP94 to proinsulin is still unknown. Based on our results and earlier papers, we have the basis to hypothesize that oxidized ASNA-1 (non-TAP targeting function) could be a co-chaperone that interacts with ENPL-1, which is required for proinsulin binding. ASNA-1 might support the binding of ENPL-1 to proinsulin or it might take part in retrograde transport to bring back the ENPL-1 to the ER.

Paper IV: Survival Motor Neuron protein (SMN-1) regulates neuropeptide secretion with Gemin2/SMI-1, independently of its Tudor domain.

RESULTS

To study the localization of SMN-1 in the organism we analyzed SMN-1::mNeonGreen and found that SMN-1 localized widely in the somatic and germ cells. Previous studies showed that loss of *smn-1* in *smn-1(ok355)* mutants resulted in growth arrest and locomotor dysfunctions (209). To confirm these results we analyzed a new mutant *syb2211*, where only the *smn-1* gene was deleted without affecting neighboring genes. The deletion resulted in the L4 arrest similar to that seen in the *ok355* deletion mutant. Additionally, by using the auxin-mediated knockdown, we depleted the SMN-1 protein from all somatic cells. This depletion resulted in growth defect and sterility. Taken together, we showed here that SMN-1 is required for the growth and development of *C. elegans* by acting only in the soma.

Previous findings showed that the SMA mice models displayed defective glucose metabolism and pancreas development (229,230). Those findings gave us a basis to study the role of SMN-1 in *C. elegans* and its function in the regulation of insulin secretion. To address that we analyzed the IIS status of the *smn-1* mutants, by scoring the nuclear and cytoplasmic subcellular localization of the DAF-16::GFP transcription factor (45). The analysis showed that the nuclear localization of DAF-16::GFP was detected in significantly more animals lacking *smn-1* gene, compared to *smn-1(+)* control. Additionally, we analyzed the ability of *smn-1* mutants to synergize with the TGF-ß pathway, by making the *smn-1;daf-2* double mutants and measuring the Daf-c phenotype (33). The analysis showed that the dauer formation of the double mutant was significantly increased compared to each single mutant. Taken together, our study showed that the *smn-1* gene influences the IIS pathway. Knowing that the IIS pathway was defective in *smn-1* mutants, we asked whether the neuropeptide and ILP secretion was disrupted as a consequence of the loss of *smn-1*. To address that we first analyzed the secretion of non-insulin neuropeptide ANF::GFP (108) and found that its secretion was affected. Next, we analyzed the secretion of SS::GFP which was used to measure the general secretion ability of *C. elegans* and we detected a significant defect in the secretion, which was consistent which previously published data (212). Furthermore, we wanted to measure the DAF-28::GFP/insulin secretion ability of *smn-1(ok355)* mutants, however, the secretion of DAF-28::GFP

could only be measured in adult animals and since *smn-1(ok355)* animals were arrested in L3/L4 stage we were not able to perform that. Instead, we knocked down the SMN-1 from somatic cells of adult animals, after coelomocytes are born and we measured DAF-28::GFP secretion and SS::GFP secretion and uptake by coelomocytes. Our analysis showed that insulin secretion and general secretion were affected when SMN-1 was knocked down from the somatic cells in adults. Taken together, our analysis showed that the *smn-1* regulates insulin signaling and general secretion processes of *C. elegans.*

Since we observed the insulin secretion defect in *smn-1* mutants, we wanted to further know whether the insulin expression and localization were affected. To address that we performed western blot and qPCR analysis to examine DAF-28::GFP protein and *daf-28* mRNA levels. Both gene and protein expression were unchanged compared to controls. Normally DAF-28::GFP is expressed and localized in the cell body, exons and dendrites of ASI and ASJ neurons (86,233) however, in *smn-1(ok355)* and *smn-1(syb2211)* mutants the localization of DAF-28::GFP changed. It was now present in additional cells, which we describe as non-neuronal, extra cells. This cell was likely an excretory gland cell based on the shape and position in the terminal bulb of the pharynx. More analysis is needed to confirm those findings and determine whether *daf-28* is now expressed in extra cells or is taken up by the gland cells. Taken together, we concluded that the loss of *smn-1* affected the localization of the DAF-28/insulin, which could contribute to the defect in insulin secretion regulation.

SMI-1/Gemin2 is a conserved, interacting partner of SMN-1 (215). Their interaction is required for the assembly of small nuclear ribonucleoproteins and the splicing of pre-mRNA (249). We wished to determine whether the splicing function of SMN-1 was responsible for the IIS defect. To do this we looked for overlaps in phenotype between SMN-1 and SMI-1. Firstly, we analyzed the *smi-1(syb3918)* mutants, which developed to adulthood, however, they were sterile similar to two *smn-1* mutants. Secondly, we analyzed the mKate2::SMI-1 in order to look for the localization of the protein. It was found in the head region and mainly in the germline. Confocal analysis showed that SMN-1::mNeonGreen and mKate2::SMI protein co-expressed in the germline of the animal. We wanted to know whether the SMN-1 localization was affected by the loss of *smi-1*. We detected abnormal distribution of SMN-1::mNeonGreen in *smi-1(syb3918)* mutants, mostly visible in the germline. Hoechst-33342 staining of the germline nuclei indicated that the SMN-1::mNeonGreen localized around the nucleus and formed intense speckles around the nucleus of the germline cells. Taken together, we

concluded that the localization of the SMN-1 was affected by the loss of its interacting partner, SMI-1 and this observation provided further indication that the two proteins interact.

Since the loss of *smi-1* affected the localization of SMN-1 we wanted to know whether the two interacting proteins had the DAF-28/insulin secretion defect. First, we analyzed the general secretion in *smi-1* mutants. By using SS::GFP as an assay we did not observe any changes in secretion of this protein, which was in contrast to the observation on *smn-1* mutants. However, the analysis of ANF::GFP and DAF-28::GFP in *smi-1* mutants indicated that the neuropeptide secretion was affected since neither of the neuropeptides was detected in coelomocytes. Taken together, we reported here that the *smi-1* mutants shared a similar phenotype with *smn-1* mutants towards neuropeptide secretion, however, the *smi-1* mutants did not exhibit any general secretion problems. This result demonstrated a difference between *smi-1* and *smn-1* mutants.

DISCUSSION

The aspect of SMN1 and the consequences of the deletion of *smn-1* gene have been mainly studied to understand the pathology of motor neuron degeneration and the consequences related to muscular atrophy (250). Recent publications indicated that loss of *smn1* had not only been associated with muscular atrophy but could be correlated with other functions of SMN1, not SMA related (251).

It has been shown that the loss of *smn1* resulted in the glucose metabolism defect and pancreatic composition defects in examined intermediate SMA mice model (229). These defects in pancreatic development in SMA mice models were discovered not to be related to the canonical SMA pathogenesis since examined mice did not have SMA hallmarks, but had already changes in pancreas composition (230). Those findings also showed that the changes in glucose metabolism led to diabetes, which was observed in their models and SMA patients (252). In our study, we used a mutant with deletion of *smn-1* gene, which was reported to affect also the locomotion of the animal, a substantial hallmark of SMA pathology (209). To understand further whether the inhibited neuropeptide secretion is correlated to neuromuscular dysfunction we could test the intermediate *smn-1* models which do not exhibit strong muscular defects. It has been shown that the *smn-1(cb131)* mutant, a novel *smn-1* allele, exhibits a milder defect equivalent to type III SMA (210). Analysis of whether this mutant exhibits a strong neuropeptide secretion defect could help us understand further the role of SMN-1in this process.

Additional, it has been shown before that the SMN binds to the α-subunit of the COPI vesicles (253). A COPI system is required for the anterograde transport of the proteins from the Golgi to the ER (254). Loss of *smn-1* and its impact on the neuropeptide secretion could be a consequence of the affected retrograde and anterograde transport. It has been shown that the loss of COPI proteins impacted insulin secretion in pancreatic β-cells (255).

Additionally, we hypothesize that the defect in insulin secretion of *smn-1* and *smi-1* mutants could be because of the defect in insulin receptor DAF-2. To test that hypothesis, we plan to analyze the *daf-2* expression level, localization and splicing in *smn-1* and *smi-1* mutants.

Taken together, our analysis indicated that using *C. elegans* we can model not only the motor neuron function of *smn-1*, but also the metabolic dysfunctions which are prominent in SMA patients. Additionally, our data showed that the *smi-1/gemin2* has a role in the regulation of insulin secretion, which indicates that the Gemin2 should be investigated for the potential pancreatic dysfunction role in humans.

5. Conclusions

In **Paper I** we established a novel role of ENPL-1 in the promotion of insulin secretion. We showed that ENPL-1 was broadly localized in the animal and that it was regulating insulin signaling and secretion. Our analysis showed that the high ER stress did not contribute to the insulin secretion defects of *enpl-1* mutants. We showed that ENPL-1 interacted with pro-insulin via the client binding domain and that the insulin processing was affected by the loss of *enpl-1*.

In **Paper II** we separated the multiple roles of ASNA-1. We showed that worm ASNA-1 was found in both oxidized and reduced states. Our analysis separated insulin secretion and tail-anchored protein insertion functions of ASNA-1 based on the analysis of the WRB-1 receptor of ASNA-1. We showed that loss of *wrb-1* did not affect the insulin secretion however, it affected the TA protein insertion and cisplatin response. Additionally, our results showed that increased oxidation of ASNA-1 led to TAP insertion defects and cisplatin sensitivity and that the cisplatin caused high oxidization of ASNA-1.

In **Paper III** we analyzed the mutual role of ASNA-1 and ENPL-1. We showed that ASNA-1 present in neurons regulated insulin secretion and that interaction between ASNA-1 and ENPL-1 took place in neurons of *C. elegans*. We discovered that ASNA-1 and ENPL-1 interaction was dependent on the presence of pro-insulin and that oxidized ASNA-1 likely interacted with ENPL-1. Our analysis showed that ASNA-1 interacted with ENPL-1 independently of its client binding domain. Our data showed that loss of *asna-1* disrupted the ER and Golgi trafficking and that the loss of *enpl-1* disrupted ASNA-1 subcellular localization.

In **Paper IV** we identified the novel role of *smn-1* in the regulation of neuropeptide secretion. We showed that loss of *smn-1* affected the growth and development of *C. elegans.* Our analysis showed that loss of *smn-1* contributed to the defect in insulin signaling and secretion and affected the distribution of DAF-28/insulin. Additionally, our data showed that loss of *smi-1* affected the subcellular localization of SMN-1 and also affected the neuropeptide secretion.

6. Future Perspective

In my thesis, I demonstrated the function of three proteins in the regulation of insulin secretion. Our observations showed that loss of any of these genes resulted in inhibited insulin secretion.

In **Paper I,** we studied the mechanism of ENPL-1 function towards a proinsulin. ENPL-1 binds the DAF-28/proinsulin via the client-binding domain and it is required for its expression and maturation. However, how exactly the abundance and maturation of the proinsulin are inhibited in the *enpl-1* mutants still remains unknown. One of the ideas is to perform a global proteomic analysis of *enpl-1* mutants and analyze the affected candidates. This could help with the initial identification of pathways and proteins that are being disrupted. A recent publication has identified the upregulated proteasome activity upon *grp94* knockdown, which contributed to the degradation of proinsulin in pancreatic β-cells. That is why this type of analysis could help identify more of such pathways. Additionally, I would like to analyze the subcellular localization of the insulin/DAF-28 in the cell and look for the changes in the *enpl-1* mutants. This could help with a better understanding of the ENPL-1 role towards insulin.

In **paper II** we showed that the ASNA-1 regulates different functions based on its redox state. The paper focused more on the ASNA-1 role in tail-anchored protein insertion and cisplatin response, rather than insulin secretion function. Our initial findings suggested that the oxidized ASNA-1 could be the form responsible for maintaining proper insulin secretion function. To build further on this aspect, I would like to analyze the localization of ASNA-1 in the cell and address whether the subcellular localization is changing when ASNA-1 is in the oxidized state compared to a reduced state. Additionally, I would like to look for small-molecule drugs that could lock ASNA-1 in the oxidized or reduced form and analyze how this permanent shift would affect its functions. Those types of analysis could be interesting for the development of **manuscript III**, where we showed that ASNA-1 interacts with ENPL-1 in the presence of proinsulin and likely is required for maintaining proper insulin secretion. Our preliminary data showed that oxidized ASNA-1 binds more to ENPL-1. Now, I would like to examine which domains of both proteins are required for the interaction. Our data showed that the interaction takes place in insulin-expressing neurons, the next step is to analyze the exact subcellular localization of the binding in the cell.

In **manuscript IV** we began to address the mechanism of SMN-1 function towards the regulation of neuropeptide secretion. To address the global defect resulting from the loss of *smn-1,* I would like to analyze the splicing defect of all the genes in the mutants. This analysis would be beneficial to distinguish which phenotypes of *smn-1* are caused by the incorrect splicing and which ones are caused by the non-splicing roles. The analysis of the splicing of insulin/DAF-28 and insulin receptor/DAF-2 would be of great interest to the project. Additionally, it would be interesting to replace the worm SMN-1 and insert human SMN1 to humanize the worm, to be able to study the disease associated mutations that potentially could affect insulin secretion or development.

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