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1. The author was involved in designing the research, performed hypothalamus RNA-sequencing, analyzed the data with S. Pajusalu, performed all the gene expression analysis, prepared figures and wrote the manuscript and handled correspondence.

2. The author was involved in research design, performed all the experiments, analyzed data and interpreted the results of experiments, wrote the manuscript and handled the correspondence.

3. The author was involved in designing the experiments, interpreting the data and participated in writing the manuscript.

4. The author was involved in designing the experiments, performed all the experiments, analyzed and interpreted the data and participated in writing the manuscript.

# ABBREVIATIONS

| | |
|---|---|
| ATF4 | activating transcription factor 4 |
| ATF6 | activating transcription factor 6 |
| AUC | area under curve |
| AVP | arginine vasopressin |
| *Avpr1a* | arginine vasopressin receptor subtype V1a gene in mouse |
| *Avpr1b* | vasopressin receptor subtype V1b gene in mouse |
| BW | body weight |
| CHOP | CCAAT/enhancer-binding protein (C/EBP) homologous protein (alternative name DDIT3, DNA damage-inducible transcript 3) |
| ER | endoplasmic reticulum |
| FDR | false discovery rate |
| GTT | glucose tolerance test |
| HFD | high fat diet |
| HSPA5 | heat shock 70 kDa protein 5 (alternative names BIP, binding immunoglobulin protein, or GRP78, 78 kDa glucose-regulated protein) |
| IRE1 | inositol-requiring enzyme 1 |
| JNK | c-Jun N-terminal kinase (alternative name MAPK8, mitogen-activated protein kinase 8) |
| $K_{ATP}$ | ATP-sensitive $K^+$ channel |
| KRBH | Krebs-Ringer solution with BSA |
| LFD | low fat diet |
| LogFC | log2 fold change |
| MAOB | monoamine oxidase B |
| RNA-seq | RNA-sequencing |
| RT-qPCR | quantitative real-time PCR |
| T2D | type 2 diabetes |
| Tol | tolbutamide |
| TRP | transient receptor potential channel family |
| TRPM5 | melastatin-related transient receptor potential subfamily member 5 protein |
| *Trpm5* | melastatin-related transient receptor potential subfamily member 5 gene in mouse; |
| *Trpm8* | transient receptor potential cation channel, subfamily M, member 8 gene in mouse |
| *Trpv3* | transient receptor potential cation channel, subfamily V, member 3 gene in mouse |
| UPR | unfolded protein response |
| *WFS1* | wolframin-1 gene in human |
| *Wfs1* | wolframin-1 gene in mouse |
| WFS1 | wolframin-1 protein |

| | |
|---|---|
| Wfs1HZ | *Wfs1* heterozygous mice |
| Wfs1KO | WFS1-deficient mice (mice homozygous for *Wfs1* mutation) |
| WFS | Wolfram syndrome |
| WT | wild-type mice |
| XBP1 | X-box binding protein 1 |

# 1. INTRODUCTION

Wolfram syndrome (WFS, OMIM 222300) is a rare progressive and neurodegenerative disorder with autosomal recessive inheritance. The disease is caused by mutations in the wolframin-1 gene (*WFS1*). The acronym DIDMOAD summarizes the main symptoms of the disorder: Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, Deafness (Barrett *et al.*, 1995, Barrett and Bundey, 1997, Inoue *et al.*, 1998, Kellner *et al.*, 1994, Strom *et al.*, 1998, Wolfram and Wagener, 1938).

The *WFS1* gene is composed of 8 exons and the first one is non-coding (Inoue *et al.*, 1998). As exon 8 is the largest, the majority of the mutations have been found in the transmembrane and C-terminal domain coding region. WFS is a heterogeneous disease, because of over a few hundred mutations have been found and the symptoms' profile is dependent on the mutation type and location (Cryns *et al.*, 2003, De Heredia *et al.*, 2013, Rigoli *et al.*, 2018). WFS1 is a hydrophobic glycoprotein with nine transmembrane segments, localizing mainly to the endoplasmic reticulum (ER) (Hofmann *et al.*, 2003, Inoue *et al.*, 1998, Philbrook *et al.*, 2005, Takeda *et al.*, 2001). WFS1 is expressed in several tissues, for example pancreatic β-cells, and in the brain (Fonseca *et al.*, 2010, Hofmann *et al.*, 2003, Inoue *et al.*, 1998, Ishihara *et al.*, 2004, Strom *et al.*, 1998). WFS1 expression in the limbic structures and hypothalamus explains endocrine regulation, cognitive and emotional behavior disabilities associated with WFS (Kato *et al.*, 2008, Luuk *et al.*, 2008, Takeda *et al.*, 2001).

WFS1 is important for the maintenance of ER homeostasis, however, the exact molecular function is still not fully elucidated. WFS1 has been found to be participating in membrane transport, protein processing, ER $Ca^{2+}$ level regulation and unfolded protein response (UPR) (Fonseca *et al.*, 2005, Fonseca *et al.*, 2010, Hatanaka *et al.*, 2011, Hofmann and Bauer, 2006, Osman *et al.*, 2003, Takeda *et al.*, 2001). WFS1 has been shown to negatively regulate UPR, because all three UPR pathways are activated in case of WFS1 dysfunction (Fonseca *et al.*, 2005, Fonseca *et al.*, 2010, Yamada *et al.*, 2006). In addition, WFS1 has been shown to regulate $Ca^{2+}$ signaling, affecting the ER $Ca^{2+}$ levels and consequently cell apoptosis (Hara *et al.*, 2014, Nguyen *et al.*, 2020, Takei *et al.*, 2006, Tan *et al.*, 2006, Toppings *et al.*, 2018). WFS1-deficient β-cells and neurons have reduced levels of $Ca^{2+}$ in the ER and increased $Ca^{2+}$ levels in cytosol, which is shown to promote cell death (Hara *et al.*, 2014, Lu *et al.*, 2014, Takei *et al.*, 2006).

The ER has many roles in the cell including regulating protein folding and transport, lipid biosynthesis and calcium homeostasis. ER is fundamental for normal cell physiology and overall health. There is growing information that pathological conditions interfering with ER homeostasis cause chronic activation of the UPR. Chronic ER stress and UPR contribute more to the pathogenesis of several diseases, including cancer, liver and neurodegenerative disorders and type 2 diabetes (Hoozemans *et al.*, 2012, Ilieva *et al.*, 2007, Xiang

*et al.*, 2017) and insulin-dependent diabetes (Cardozo *et al.*, 2005, Gwiazda *et al.*, 2009, Hoozemans *et al.*, 2012, Ilieva *et al.*, 2007, Ozcan and Tabas, 2012, Xiang *et al.*, 2017). WFS1 is normally upregulated in case of ER stress, therefore its deficiency promotes unresolved ER stress and cell apoptosis, leading to progression symptoms characteristic to WFS (Fonseca *et al.*, 2005, Fonseca *et al.*, 2009, Fonseca *et al.*, 2010, Ishihara *et al.*, 2004).

Because WFS is a rare disorder, several rodent models have been created in order to study the underlying molecular mechanisms. The WFS mouse model used in this dissertation was created by replacing most of the *Wfs1* exon 8 producing a truncated WFS1 protein (Koks *et al.*, 2009, Luuk *et al.*, 2009). The current study aimed to find possible transcriptomic and metabolic changes due to *Wfs1* deficiency that may contribute to the development of WFS phenotype. RNA-sequencing was used to find transcriptomic changes in the hypothalamus, hippocampus, and pancreatic islets of Langerhans in WFS1-defiecient mice. As insulin-dependent diabetes is the main symptom of WFS, insulin secretion was analyzed in isolated pancreatic islets. In addition, metabolic function of WFS1-deficient mice was studied. Metabolic disorders, like type 2 diabetes, have been associated with chronically elevated ER stress due to chronic overfeeding, for example (Bhattarai *et al.*, 2020, Fernandes-Da-Silva *et al.*, 2021). Several studies have correlated mutations in *WFS1* with an increased risk of developing type 2 diabetes (Cheurfa *et al.*, 2011, Florez *et al.*, 2008, Franks *et al.*, 2008, Minton *et al.*, 2002, Sandhu *et al.*, 2007, Van Hoek *et al.*, 2008). Therefore, the susceptibility of *Wfs1* heterozygous mice on developing metabolic disturbances induced by high fat diet was studied.

# 2. REVIEW OF THE LITERATURE

## 2.1. Wolfram syndrome

Wolfram syndrome (WFS, OMIM 222300) is a rare autosomal recessive neurodegenerative and progressive congenital disorder caused by mutations in the wolframin-1 gene (*WFS1*). The main symptoms of the disease are juvenile-onset diabetes mellitus, progressive optic atrophy, diabetes insipidus and deafness. The first letters of the main symptoms in the following order *Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, Deafness* combine into the acronym DIDMOAD by which WFS is also known (Barrett *et al.*, 1995, Barrett and Bundey, 1997, Inoue *et al.*, 1998, Kellner *et al.*, 1994, Strom *et al.*, 1998, Wolfram and Wagener, 1938).

The prevalence of WFS is estimated to be 1:160 000–770 000 (Barrett and Bundey, 1997, Barrett *et al.*, 1995, Rohayem *et al.*, 2011) to 1:54 478 in different ethnic groups (Lombardo *et al.*, 2014). The juvenile non-autoimmune diabetes mellitus and optic atrophy are the main criteria for WFS diagnosis (Barrett *et al.*, 1995). However, the clinical presentation of WFS is heterogeneous and depends on the type and location of the mutation. Therefore, in addition to the medical and family history, Sanger sequencing-based genetic testing is nowadays used to confirm the diagnosis (Barrett *et al.*, 1995, Urano, 2016).

In case of WFS, the non-autoimmune insulin-dependent diabetes often occurs before 15 years of age, usually around the age of 5-6 (Barrett *et al.*, 1995, Rigoli *et al.*, 2018, Tranebjaerg *et al.*, 2009). The course of the WFS diabetes is considered milder than in case of type 1 diabetes mellitus, because diabetic ketoacidosis and microvascular complications are rare. The WFS patients are not usually obese. In general, WFS patients require lower insulin doses compared to autoimmune type 1 diabetes patients as the degeneration of pancreatic islets generates disturbances in insulin secretion leading to gradual degeneration (Cano *et al.*, 2007, Tranebjaerg *et al.*, 2009, Urano, 2016). The degeneration or depletion of pancreatic β-cells is due to non-autoimmune mechanism (Karasik *et al.*, 1989), because postmortem examinations of WFS patients have showed loss of β-cells or atrophy of the pancreatic islets, although the exocrine portion of the gland was normal or with focal areas of fibrosis (Rigoli *et al.*, 2020, Wolfram and Wagener, 1938). Diabetes development depends on the mutation type and location of the mutation in the *WFS1* gene, whether it causes a total or partial function loss (Rohayem *et al.*, 2011). Genome-wide studies have linked several *WFS1* mutations as a risk factor for developing type 2 diabetes (Cheurfa *et al.*, 2011, Florez *et al.*, 2008, Franks *et al.*, 2008, Minton *et al.*, 2002, Sandhu *et al.*, 2007, Van Hoek *et al.*, 2008).

Optic atrophy in case of WFS is progressive and typically diagnosed before 15 years of age, usually around the age of 11 (Barrett *et al.*, 1995, Tranebjaerg *et al.*, 2009). It is characterized by a progressive decrease in visual acuity with loss of color vision and peripheral vision, which eventually leads to blindness. Other visual system abnormalities, such as nystagmus, maculopathy, retino-

pathy and cataracts, have also been observed in WFS patients (Delvecchio *et al.*, 2021, Hu *et al.*, 2021, Tranebjaerg *et al.*, 2009, Zmyslowska *et al.*, 2017). In addition, corneal abnormalities similar to keratoconus have been noted (Waszczykowska *et al.*, 2020a, Waszczykowska *et al.*, 2020b). Retinal thinning has been shown to be a valid marker assessing disease progression (Hoekel *et al.*, 2014, Hu *et al.*, 2021, Zmyslowska *et al.*, 2015).

Another prevalent manifestation of WFS is diabetes insipidus that affects about 70% of WFS patients and occurs mostly in the second decade of life (Barrett *et al.*, 1995, Urano, 2016). Urinary tract complications and neurogenic bladder are also clinical challenges that are common for WFS. Problems with obstruction of the ducts between the kidneys and bladder, high-capacity atonal bladder, disrupted urination, bladder sphincter dyssynergia, and difficulty controlling urine flow have been noted (Barrett *et al.*, 1995). In addition, reproductive endocrine dysfunctions, such as hypogonadism, or menstrual abnormalities, are often encountered (Rigoli *et al.*, 2018).

Growth retardation and hypothyroidism have also been reported and associated with hypothalamus-pituitary axis dysfunction. In addition, problems with gait, coordination and balance have been described in WFS patients (Barrett *et al.*, 1995, Rigoli *et al.*, 2018, Tranebjaerg *et al.*, 2009).

Around 60% of WFS patients develop sensorineural deafness. The severity of the hearing loss may vary from deafness beginning at birth to mild hearing loss beginning in adolescence that progressively deteriorates (Barrett *et al.*, 1995, Barrett and Bundey, 1997, Rigoli *et al.*, 2018).

WFS patients may also have several neurological abnormalities that occur later such as anosmia, seizures, dysarthria, dysphagia, mental retardation and nystagmus (Barrett *et al.*, 1995). Moreover, suicidal behavior and psychiatric illnesses, like depression and psychosis, are common for WFS patients (Swift *et al.*, 1990, Swift *et al.*, 1991, Swift *et al.*, 1998). *WFS1* heterozygous mutation carriers have a higher possibility of psychiatric hospitalization, primarily due to depression (Swift *et al.*, 1998, Swift and Swift, 2000).

The neurological abnormalities are progressive, leading to general brain atrophy. The cerebellum, pons, and medulla are the most affected brain regions (Barrett *et al.*, 1995, Pakdemirli *et al.*, 2005). The prognosis of this syndrome is currently poor, because patients usually die around 30 years of age (range 25-49 years). The predominant cause is central respiratory failure due to brain stem atrophy (Barrett *et al.*, 1995, Barrett and Bundey, 1997, Rigoli *et al.*, 2020, Scolding *et al.*, 1996, Urano, 2016).

## 2.2. WFS1 gene

*WFS1* is a nuclear gene with a total size of 33.4 kb (Inoue *et al.*, 1998, Polymeropoulos *et al.*, 1994, Strom *et al.*, 1998). The loss-of-function mutations in wolframin-1 (*WFS1*) gene cause WFS. *WFS1* is located on the short arm of chromosome 4 (4p16.1). The gene has 8 exons of which the first one is non-

coding (Inoue *et al.*, 1998). Exon 8 is the largest (2.6 kb) and contains about 60% of the protein coding sequence, encoding for the transmembrane and C-terminal domain of WFS1. Majority of the mutations have been found in exon 8. Over 200 mutations have been described in *WFS1*, causing WFS to be a very heterogeneous disease (Cryns *et al.*, 2003, De Heredia *et al.*, 2013, Rigoli *et al.*, 2018). In mice the *Wfs1* is located on the long arm of the chromosome 5 (5qB3) and the nucleotide sequence is very homologous to the human *WFS1* gene sequence with 83% identity at the nucleotide level (Strom *et al.*, 1998).

## 2.3. WFS1 protein and its functions

The *WFS1* gene encodes wolframin (WFS1) protein with 890 amino acid residues and a molecular mass of around 100 kDa. WFS1 is a hydrophobic glycoprotein with nine transmembrane segments (Hofmann *et al.*, 2003, Inoue *et al.*, 1998). WFS1 has been shown to localize primarily to the endoplasmic reticulum (ER) in the cells. The N-terminal part of WFS1 is in the cytoplasm and the C-terminal part in the ER lumen (Hofmann *et al.*, 2003, Inoue *et al.*, 1998, Philbrook *et al.*, 2005, Takeda *et al.*, 2001). The human and mouse WFS1 amino acid sequences have an 87% identity (Inoue *et al.*, 1998, Strom *et al.*, 1998).

The molecular mechanism and function of WFS1 are not fully elucidated, but it has been shown that WFS1 is important for the maintenance of ER homeostasis. Due to its localization to the ER in the cells, WFS1 has been found to be participating in membrane transport, processing proteins and/or regulation of the calcium homeostasis and unfolded protein response (UPR) signaling pathway in the ER (Fonseca *et al.*, 2005, Fonseca *et al.*, 2010, Hatanaka *et al.*, 2011, Hofmann and Bauer, 2006, Osman *et al.*, 2003, Takeda *et al.*, 2001).

The expression of WFS1 is highest in brain, pancreatic β-cells, heart and muscles. Smaller level of expression is seen in kidneys, liver and spleen (Fonseca *et al.*, 2010, Hofmann *et al.*, 2003, Inoue *et al.*, 1998, Ishihara *et al.*, 2004, Strom *et al.*, 1998). WFS1 is expressed in brain regions related to the neurological impairments of WFS. Its expression in the limbic structures and hypothalamus explains endocrine regulation, cognitive and emotional behavior disabilities associated with WFS. The expression has been noticeable in the cerebral cortex and hippocampus, but also in amygdala, ventral striatum (nucleus accumbens and olfactory tubercle), thalamus and in several hypothalamic nuclei (Kato *et al.*, 2008, Luuk *et al.*, 2008, Takeda *et al.*, 2001). WFS1 expression in pancreas is localized to the β-cells of islets of Langerhans, and is absent in other islet cells and exocrine pancreas (Inoue *et al.*, 1998, Ishihara *et al.*, 2004).

## 2.4. WFS1-deficient rodent models

As WFS is a very heterogeneous disease, there are also rodent models in addition to cell line models to investigate the causal interactions and treatment options. However, each model is different depending on which part of the gene is disrupted.

One of the first mouse models had the second exon of *Wfs1* disrupted causing loss or severely impaired WFS1 function (Ishihara *et al.*, 2004). The *Wfs1* exon 2 knock-out mice displayed severe glucose intolerance and disturbed insulin secretion, although insulin tolerance did not change, already at 2 weeks of age. Around 2 months of age progressive loss of pancreatic β-cells was observed due to ER stress linked apoptosis. It was also observed that the diabetic symptoms were present only in 129SVEV x C57BL/6 F2 genetic background and further backcrossing with C57BL/6 reduced the observed WFS phenotype (Ishihara *et al.*, 2004). Studying the behavior of *Wfs1* exon 2 mutant mice showed that these mice had shorter escape latency in the habituation phase of the passive and active avoidance tests. In addition, they displayed increased freezing in the training phase of the fear conditioning test and longer latency to find the platform in the learning phase of the Morris water maze test (Kato *et al.*, 2008).

The other mouse model is a conditional knock-out of *Wfs1* exon 8 in the pancreatic β-cells in a 129SVJ genetic background (Riggs *et al.*, 2005). These mice had functional WFS1 in all the other tissues except pancreatic β-cells. Around 4 months of age these mice displayed glucose intolerance and insulin deficiency. In addition, their body weight was lower compared to wild-type controls. By 24 months of age the mice had significantly increased apoptosis of β-cells and decreased β-cell mass (Riggs *et al.*, 2005). The mouse model created by Riggs *et al.* (2005) showed earlier development of metabolic disturbances then the model by Ishihara *et al.* (2004).

Mice used in this dissertation were created by replacing most of the *Wfs1* exon 8 with an NLSLacZNeo expression cassette, which caused a truncated WFS1 protein lacking the amino acids 360–890 (Koks *et al.*, 2009, Luuk *et al.*, 2009). Starting from 2 months of age the mice had lower body weight, decreased insulin secretion and severe glucose intolerance, later they developed also increased fasted blood glucose levels (Koks *et al.*, 2009, Luuk *et al.*, 2009, Noormets *et al.*, 2011). In addition, the male mice had more pronounced metabolic disturbances and reduced fertility compared to the females (Luuk *et al.*, 2009, Noormets *et al.*, 2011). The WFS1-deficient males had reduced plasma insulin and leptin levels, but increased proinsulin/insulin ratio in addition to hyperglycemia and significant weight loss (Noormets *et al.*, 2011, Noormets *et al.*, 2014). It has been shown that their energy metabolism and thyroid function around 11–13 weeks of age are rather normal, regardless of lower caloric intake and increased weight loss in the metabolic cages (Noormets *et al.*, 2014). The reason for that might have been the impaired behavioral adaption mechanisms instead of metabolic state changes, because WFS1-deficient mice also display

impaired behavioral adaptation in new and stressful environment (Luuk *et al.*, 2008, Luuk *et al.*, 2009) together with dysfunctional dopaminergic and serotonergic signaling (Reimets *et al.*, 2016, Visnapuu *et al.*, 2013a).

Besides mouse models there is also a *Wfs1* loss-of-function rat model, in which the exon 5 is mutated (Plaas *et al.*, 2017). By the 12 months of age, the *Wfs1* exon 5 mutant rats develop severe glucose intolerance, insulin-dependent diabetes, glycosuria and hyperglycemia and have reduced body weight. Furthermore, these rats display optic nerve atrophy and medullary degeneration and the ER stress marker levels are elevated in the pancreas and brainstem (Plaas *et al.*, 2017).

## 2.5. WFS1 in ER stress

Endoplasmic reticulum (ER) is a cellular organelle with many functions. It is important for protein synthesis, sorting and transport and storing $Ca^{2+}$ ions (Ariyasu *et al.*, 2017). Various physiological processes can increase the level of protein folding and posttranslational modifications happening in the ER, for example, the higher need for insulin after eating, infections or mutant protein expression. If the folding capacity of the ER exceeds its limits, the unfolded or misfolded proteins start to accumulate inside the ER lumen. This causes cells to exhibit a condition defined as ER stress. In case of ER stress a network of signaling pathways is activated, called the unfolded protein response (UPR). The prime function of UPR is to mitigate ER stress and generate proteins for cell survival as a beneficial coping mechanism. However, chronic and high ER stress due to pathological conditions leads to cell apoptosis (Ozcan and Tabas, 2012, Riahi *et al.*, 2018, Rouzier *et al.*, 2017, Toppings *et al.*, 2018).

UPR has three main signaling pathways defined by the three primary proteins: inositol-requiring protein 1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6). Activation of these proteins can lead to either survival-adaptive or death responses. Under physiological conditions, the ER chaperone heat shock 70 kDa protein 5 (HSPA5), also known as BIP (binding immunoglobulin protein) or GRP78 (78 kDa glucose-regulated protein), binds luminal domains of IRE1, ATF6 and PERK to keep them in an inactive state. When incorrectly folded proteins accumulate in the ER lumen, HSPA5 is released from these complexes to help with the folding of accumulated proteins (Gardner and Walter, 2011, So, 2018). Under ER stress, activation of ATF6 leads to the activation of X-box binding protein 1 (XBP1) and production of chaperones GRP94 and HSPA5. Activated PERK phosphorylates the translation initiation factor elF2α, inhibiting translation, but activating C/EBP homologous protein (CHOP). Activated IRE1 helps to produce functional XBP1. Fully functional XBP1 induces UPR target genes expression that are involved in ER protein folding and degradation. HSPA5 expression is also regulated by IRE1 (Ariyasu *et al.*, 2017).

ER stress markers' levels from all three UPR pathways are increased in the absence of WFS1, indicating that WFS1 may be a negative regulator of UPR

(Fonseca *et al.*, 2005, Fonseca *et al.*, 2010, Yamada *et al.*, 2006). WFS1 is shown to downregulate the activity of ATF6 and its downstream targets (Fonseca *et al.*, 2010). Under physiological stress and in normal healthy cells, WFS1 inhibits ATF6 activation, leading it to ubiquitination and proteasomal degradation. In case of defective WFS1, ATF6 is hyperactivated. This causes activation of cellular apoptosis promoting genes, like CHOP and ATF4, but also HSPA5 and XBP1 (Fonseca *et al.*, 2005, Fonseca *et al.*, 2009, Lipson *et al.*, 2006).

In the pancreatic β-cells of WFS1-deficient mice it has been shown that the expression of HSPA5, GRP94 and spliced XPB1 is elevated (Yamada *et al.*, 2006). In addition, WFS1 expression is shown to be increased during insulin secretion, suggesting that WFS1 is an important component of proinsulin folding and processing in the β-cells' ER (Fonseca *et al.*, 2005, Fonseca *et al.*, 2009, Fonseca *et al.*, 2010, Lipson *et al.*, 2006).

In addition, WFS1 may regulate $Ca^{2+}$ signal transduction processes too, affecting the storage of cellular ER $Ca^{2+}$ levels and, therefore, apoptosis of the cells (Hara *et al.*, 2014, Nguyen *et al.*, 2020, Takei *et al.*, 2006, Tan *et al.*, 2006, Toppings *et al.*, 2018). WFS1-deficient β-cells and neurons have reduced levels of $Ca^{2+}$ in the ER and increased $Ca^{2+}$ levels in cytosol, which is shown to promote cell death (Hara *et al.*, 2014, Lu *et al.*, 2014, Takei *et al.*, 2006). WFS1 has also been reported to influence the function of sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA), which is essential for ER $Ca^{2+}$ homeostasis in pancreatic β-cells (Cardozo *et al.*, 2005, Zatyka *et al.*, 2015). Furthermore, WFS1 has been shown to interact with the ion pumps $Na^{+}$ $K^{+}$ ATPase and vacuolar-type $H^{+}$ ATPase supporting the role of WFS1 in protein folding, biosynthesis and secretion (Gharanei *et al.*, 2013, Zatyka *et al.*, 2008).

It has been determined that ER perturbations and high levels of prolonged unresolved ER stress induce cell death in neurodegenerative disorders (Hoozemans *et al.*, 2012, Ilieva *et al.*, 2007, Xiang *et al.*, 2017) and insulin-dependent diabetes (Cardozo *et al.*, 2005, Gwiazda *et al.*, 2009). If WFS1 is normally upregulated in case of ER stress, then its deficiency promotes cell apoptosis, leading to progression symptoms characteristic to WFS (Fonseca *et al.*, 2005, Fonseca *et al.*, 2009, Fonseca *et al.*, 2010, Ishihara *et al.*, 2004).

## 2.6. Summary of the literature review

Wolfram syndrome (WFS) is a rare progressive neurodegenerative disorder with main symptoms of insulin-dependent diabetes and optic atrophy. Regardless of the main characteristics, WFS is a heterogeneous disease, because the symptoms' profile is dependent on the mutation type and location within the causative *WFS1* gene.

WFS1 is shown to be important for the maintenance of endoplasmic reticulum (ER) homeostasis, however, the exact molecular function is still not fully elucidated. WFS1 has been found to be participating in membrane transport,

protein processing, ER $Ca^{2+}$ level regulation and unfolded protein response (UPR). WFS1 is shown to be upregulated in case of ER stress, therefore its deficiency promotes unresolved ER stress and cell apoptosis, leading to progression symptoms characteristic to WFS. There is growing information that pathological conditions interfering with ER homeostasis cause chronic activation of the UPR. Chronic ER stress and UPR contribute more to the pathogenesis of several diseases, including neurodegenerative disorders and diabetes.

Because WFS is a rare disorder, using rodent models is the only option for biomedical and translational research. WFS1-deficient mice with the disrupted exon 8 used in this dissertation were generated in University of Tartu (Koks *et al.*, 2009, Luuk *et al.*, 2009). This model displays impaired behavioral adaptation in new and stressful environments and has widespread neurodegeneration, muscular dystrophy, retinal degeneration, diabetes and multiple neurochemical changes. This mouse model has helped to describe the pathophysiology of WFS and identify ER stress and mitochondrial dysfunction as the pathogenic mechanisms. One particular aspect of WFS is the metabolic changes that have not been studied so well in the past. The Wfs1KO mice have significantly reduced growth and body weight, severe glucose intolerance and impaired fertility. The pathophysiology of these metabolic changes has not been understood very well. Therefore, the current work aimed to get more information on the molecular mechanisms of metabolic changes. The metabolic physiology with transcriptomic analysis was combined to uncover some of the hidden strings of the metabolic pathology of the Wolfram syndrome.

# 3. AIMS OF THE STUDY

The current study aimed to find possible transcriptomic and metabolic changes due to *Wfs1* deficiency that may contribute to the development of Wolfram syndrome phenotype.

Specifically the aims were:

1. to compare transcriptomic alterations in the hypothalamus, hippocampus, and pancreatic islets of Langerhans in WFS1-defiecient mice;
2. to analyze the insulin secretion and proinsulin content in isolated pancreatic islets of WFS1-deficient mice;
3. to quantify *in vivo* thermogenesis as an estimate of metabolic function in WFS1-deficient mice;
4. to analyze susceptibility to high energy diet induced complications and possible alterations in ER stress gene expression profile in heterozygous *Wfs1* mice.

Thus, these studies aimed to find out potential molecular, cellular and metabolic connections associated with the physiological responses and mechanisms in experimental WFS1 study model.

# 4. MATERIALS AND METHODS

## 4.1. Animals (Paper I–IV)

The animal experiments described in the studies were performed with permission from Estonian National Board of Animal Experiments (No. 71, April 8th, 2011) and in accordance with the European Communities Directives (86/609/EEC and 2010/63/EU). Generation of *Wfs1* mutant (Wfs1KO, Wfs1$^{tm1Koks}$) mice has been previously described elsewhere (Koks *et al.*, 2009, Luuk *et al.*, 2008). In the first (Ivask *et al.*, 2018) and third (Ehrlich *et al.*, 2016) paper, two genotypes of littermate mice were used: wild-type (WT) and homozygotes for *Wfs1* mutation (Wfs1KO). In the second paper (Ivask *et al.*, 2016), also heterozygotes for *Wfs1* mutation (Wfs1HZ) were used. In the fourth paper (Ivask *et al.*, 2021), only Wfs1HZ and WT mice were used. All studies were performed on F2 generation male mice as Wfs1KO male mice have more pronounced metabolic disturbances compared to Wfs1KO female mice (Luuk *et al.*, 2009) and in order to reduce variation and complexity of the study. In the first and third paper the background of the mice was 129S6/SvEvTac x 129S6/ SvEvTac, but in the second and fourth one 129S6/SvEvTac x C57BL/6. In the first paper the mice were 6–7 months old at the time of the experiment, in the second 5–6 months old and in the third one 9–12 months old, in the fourth paper the starting age of mice was 2–3 months. Table 1 summarizes animal usage throughout the papers. Mice were housed in groups of 6-8 at 20±2°C under 12-h/12-h light/dark cycle with free access to food and water, unless otherwise needed for the experiment (Ehrlich *et al.*, 2016, Ivask *et al.*, 2016, Ivask *et al.*, 2018, Ivask *et al.*, 2021).

**Table 1.** Animals used*

| Paper | WT | Wfs1HZ | Wfs1KO | Age (months) | Gender | Background |
|---|---|---|---|---|---|---|
| I | 9 | – | 9 | 6–7 | male | 129S6/SvEvTac x 129S6/SvEvTac |
| II | 10 | 10 | 10 | 5–6 | male | 129S6/SvEvTac x C57BL/6 |
| III | 60 | – | 58 | 9–12 | male | 129S6/SvEvTac x 129S6/SvEvTac |
| IV | 15 | 16 | – | 2–3 | male | 129S6/SvEvTac x C57BL/6 |

* The total number of animals per genotype used in one complete paper is presented in the table.

## 4.2. RNA extraction (Paper I, II, IV)

For paper I mice were sacrificed by cervical dislocation to dissect hippocampi and hypothalami. For hippocampus dissection 4 animals and for hypothalamus 5 animals were used from both genotypes. Total RNA was isolated from hippocampus and hypothalamus using mirVana miRNA Isolation Kit (Life Technologies/Thermo Fisher Scientific) according to the manufacturer's protocol. Subsequent DNase I treatment was also performed according to manufacturers' protocol (Qiagen). The RNA quality was assessed using Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies) (Ivask *et al.*, 2018).

In paper II total RNA was isolated from islets of 4 animals in each genotype group using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol and cDNA for sequencing was synthesized using Ovation RNA-Seq System V2 (NuGEN Technologies), an input of 10 ng of total RNA was used (Ivask *et al.*, 2016).

In paper IV mice were sacrificed by decapitation to dissect heart, liver and kidneys. Total RNA was extracted using TRIzol reagent (Invitrogen/Thermo Fisher Scientific) according to the manufacturers' protocol. The RNA from pancreatic islets was isolated using RNeasy Plus Mini Kit (Qiagen). The purity and concentration of RNA samples were determined with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) (Ivask *et al.*, 2021).

## 4.3. Transcriptome analysis (Paper I, II)

In paper I for hippocampus (n=4 for both genotypes) whole transcriptome sequencing a total of 10 μg of RNA was treated with RiboMinus Eukaryote Kit for RNA-seq (Invitrogen/Thermo Fisher Scientific) to eliminate ribosomal RNA from the rest of the transcriptome. The SOLiD Total RNA-Seq Kit and 500 ng of ribodepleted RNA was used according to the manufacturer's protocol (Life Technologies/Thermo Fisher Scientific) for whole transcriptome RNA-seq library preparation. The libraries were barcoded and pooled together for the following template preparation. The sequencing was performed with SOLiD 4 platform (Life Technologies/Thermo Fisher Scientific) using paired-end DNA sequencing chemistry (50 bp forward and 35 bp reverse) (Ivask *et al.*, 2018).

For hypothalamus (n=5 for both genotypes) whole transcriptome sequencing cDNA was synthesized using Ovation RNA-Seq System V2 (NuGEN Technologies). Fifty ng of total RNA was used. SOLiD DNA Fragment library kit (cDNA input 2 μg) was used to generate libraries and quality was controlled with the Agilent Bioanalyzer 2100 and High Sensitivity DNA Kit (Agilent Technologies) before sequencing. The libraries were barcoded and pooled together for the template preparation. The template was prepared with automated SOLiD EZ Bead System and SOLiD EZ Bead E80 System Consumables (Life Technologies/Thermo Fisher Scientific). The SOLiD 5500xl System and

paired-end (75 bp forward and 35 bp reverse) chemistry for DNA sequencing were applied (Life Technologies/Thermo Fisher Scientific) (Ivask *et al.*, 2018).

In paper II for pancreatic islets (n=4 for each genotype) SOLiD DNA Fragment library kit (cDNA input 2 µg) was used to generate libraries and quality was controlled with the Agilent Bioanalyzer 2100 (Agilent Technologies) before sequencing. The libraries were marked with different barcodes and pooled together for the template preparation with automated SOLiD EZ Bead E80 System and its consumables (Life Technologies/Thermo Fisher Scientific). The SOLiD 5500xl System and paired-end (75 bp forward and 35 bp reverse) chemistry (Life Technologies/Thermo Fisher Scientific) were used for sequencing. Samples from each animal were sequenced and analyzed separately (Ivask *et al.*, 2016).

All the raw sequences have been deposited in the sequence read archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/) under the accession numbers GSE102625 (Ivask *et al.*, 2018) and GSE65929 (Ivask *et al.*, 2016).

## 4.4. Functional annotation of transcriptome (Paper I, II)

Ingenuity Pathway Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com) was used to define the functional networks of the differentially expressed genes in papers I and II. The entire datasets containing gene identifiers, corresponding false discovery rate (FDR) and $\log_2$ fold change (LogFC) were filtered to get only LogFC and FDR corrected significant genes. Statistical significance filtering was used to increase the focus and specificity of analysis. The dataset was thereafter mapped to the Ingenuity Pathways Knowledge Base. IPA then generated networks of the focus genes based on their connectivity and calculated a significance score for each network. The score indicates that the identified genes in a network are not assembled together by random chance (displayed as the negative logarithm of the *P* value). For example, a score of 2 means that there is less than 1:100 chance that the focus genes are not regulated in tandem (Ivask *et al.*, 2016, Ivask *et al.*, 2018).

## 4.5. Quantitative real-time PCR (Paper I, II, IV)

The quantitative real-time PCR (RT-qPCR) was performed using TaqMan gene expression assays and chemistry (Thermo Fisher Scientific). The used gene assays are listed in table 2. *Hprt* was used as an endogenous control in all the RT-qPCR experiments.

**Table 2.** TaqMan assays used for RT-qPCR

| Gene symbol | Gene name | TaqMan assay ID | Paper |
|---|---|---|---|
| *Hprt* | hypoxanthine phosphoribosyl transferase | Mm00446968_m1 | I, II, IV |
| *Wfs1* | wolframin | Mm01220326_m1 | I, IV |
| *Hspa5* | heat shock 70 kDa protein 5 | Mm00517691_m1 | I, IV |
| *Trpm8* | transient receptor potential cation channel, subfamily M, member 8 | Mm01299593_m1 | I |
| *Cyb5r2* | cytochrome b5 reductase 2 | Mm00623496_m1 | I |
| *Ccl28* | chemokine (C-C motif) ligand 28 | Mm00445039_m1 | I |
| *Olfr434* | olfactory receptor 47 | Mm00836760_s1 | I |
| *Sesn2* | sestrin 2 | Mm00460679_m1 | I |
| *Gfra4* | glial cell line derived neurotrophic factor family receptor alpha 4 | Mm00498382_m1 | I |
| *Avpr1a* | arginine vasopressin receptor 1A | Mm00444092_m1 | I |
| *Npm1* | nucleophosmin (nucleolar phosphoprotein B23, numatrin) | Mm02391781_g1 | I |
| *Glipr2* | glioma pathogenesis-related protein 2 | Mm01341451_m1 | II |
| *Trpm5* | transient receptor potential cation channel, subfamily M, member 5 | Mm01129032_m1 | II |
| *Gad1* | glutamate decarboxylase 1 | Mm00725661_s1 | II |
| *MaoB* | monoamine oxidase B | Mm00555412_m1 | II |
| *ApoE* | apolipoprotein E | Mm01307193_g1 | II |
| *Atf6α* | activating transcription factor 6 alpha | Mm01295317_m1 | IV |
| *Chop* | CCAAT/enhancer-binding protein (C/EBP) homologous protein | Mm00492097_m1 | IV |
| *Ire1α* | inositol-requiring enzyme 1 alpha | Mm00470233_m1 | IV |
| *Xbp1* | X-box binding protein 1 | Mm00457357_m1 | IV |
| *Atf4* | activating transcription factor 4 | Mm00515324_m1 | IV |
| *Bcl2* | B cell leukemia/lymphoma 2 | Mm00477631_m1 | IV |
| *Jnk* | c-Jun N-terminal kinase | Mm00489514_m1 | IV |
| *Casp3* | caspase 3 | Mm01195085_m1 | IV |

In paper I five hippocampal and hypothalamic samples from each genotype were analyzed three times. Total RNA from each sample was subjected to cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Life Technologies/Thermo Fisher Scientific) following the manufacturer's protocol. The total RNA input for hippocampus was 2 µg and for hypothalamus 1 µg. The expression of *Wfs1* and *Hspa5* were analyzed from both tissues. Additionally for hippocampus data *Trpm8*, *Cyb5r2*, *Ccl28*, *Olfr434* and *Sesn2* and for hypothalamus *Gfra4*, *Avpr1a* and *Npm1* were selected for validation. The ViiA 7 Real-Time PCR System (Life Technologies/Thermo Fisher Scientific) was used for analyzing (Ivask *et al.*, 2018).

In paper II the pancreatic islet samples were treated with TURBO DNA-free kit (Ambion/Thermo Fisher Scientific), according to the manufacturer's instruc-

tions to remove any possible contaminating genomic DNA. Total RNA of 10 ng from each sample was subjected to cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Life Technologies/Thermo Fisher Scientific) following the manufacturer's protocol. The expression of *Glipr2*, *Trpm5*, *Gad1*, *MaoB*, *ApoE* was analyzed using the ABI Prism 7900 HT Sequence Detection System (Life Technologies/Thermo Fisher Scientific). The same samples were analyzed two times, but three times for *Trpm5* (Ivask *et al.*, 2016).

In paper IV total RNA from each sample was subjected to cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/ Thermo Fisher Scientific) following the manufacturer's protocol. The total RNA input for heart, liver and kidney was 2 µg and for pancreatic islets 0.04 µg. The TaqMan gene expression assays used were *Wfs1*, *Hspa5*, *Atf6α*, *Chop*, *Ire1α*, *Xbp1*, *Atf4*, *Bcl2*, *Jnk* and *Casp3*. The samples were analyzed with the ViiA7 Real-Time PCR system (Applied Biosystems/Thermo Fisher Scientific) (Ivask *et al.*, 2021).

## 4.6. Insulin secretion assay (Paper II, IV)

In paper II (Ivask *et al.*, 2016) and paper IV (Ivask *et al.*, 2021) pancreatic islets were isolated as previously described (Shimomura *et al.*, 2009). Mice were sacrificed by cervical dislocation and the islets isolated by collagenase Type XI digestion (Sigma-Aldrich/Merck, final concentration 1 mg/mL). The inflated pancreas was dissected out and incubated in a 37°C water bath for 13 minutes. The tissue was washed twice with 0.2% BSA (Sigma-Aldrich/Merck) and HBSS (Sigma-Aldrich/Merck) solution. The islets were handpicked under stereomicroscope in 0.2% BSA and HBSS solution. Lastly, the islets were picked into high glucose (4.5 g/L) DMEM/Ham's F12 media (PAA/Thermo Fisher Scientific), containing 10% FBS (Gibco/Thermo Fisher Scientific), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco/Thermo Fisher Scientific) and 20 mM L-glutamine (Invitrogen/Thermo Fisher Scientific). Islets were incubated overnight at 37°C before insulin secretion assay. During the picking the number of islets was counted manually.

Before starting the insulin secretion assay the islets were incubated for 1 h at 37°C and 5% $CO_2$ in 0.2% BSA and Krebs-Ringer solution (KRBH, 140 mM NaCl, 0.5 mM $NaH_2PO_4$, 2 mM $NaHCO_3$, 3.6 mM KCl, 0.5 mM $MgSO_4$, 2.6 mM $CaCl_2 \cdot 2H_2O$, 5 mM HEPES, pH 7.4) containing 2 mM glucose. Then, islets were incubated for 1 h at 37°C and 5% $CO_2$ in selected assay solution (KRBH and 2 mM, 10 mM or 20 mM glucose or 200 µM tolbutamide and 2 mM glucose). Tolbutamide (Sigma-Aldrich/Merck) was first dissolved in 0.2% DMSO (Sigma-Aldrich/Merck) with final concentration of 200 µM. Each assay media group contained 5 islets and was in duplicate per genotype. After incubation the supernatant was collected and stored at -20°C until ELISA analysis. To determine total insulin content, insulin was extracted from the same

islets using 95:5 ethanol:acetic acid solution (Ivask *et al.*, 2016, Ivask *et al.*, 2021, Shimomura *et al.*, 2009).

Insulin concentration was determined with Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc.). Insulin amount was determined separately from secretion and islet content samples. To reduce the effect of variation in islet size on insulin secretion, the secreted amount of insulin was normalized to the content of insulin (secreted insulin divided by insulin content) (Ivask *et al.*, 2016, Ivask *et al.*, 2021).

## 4.7. Proinsulin assessment (Paper II)

In paper II proinsulin concentration was measured from islet content samples with Mouse Proinsulin ELISA Kit (Wuhan EIAab Science Co., Ltd.). Proinsulin was also determined from secretion samples, but the results were too low and did not reach the detection range. Differences in the amount of proinsulin were normalized to the number of islets used in each well. To compare proinsulin/ insulin ratio between genotypes the proinsulin concentration was divided with corresponding normalized insulin (Ivask *et al.*, 2016).

## 4.8. Metabolic studies (Paper III)

For metabolic experiments in paper III Wfs1KO mice were always used in parallel with their WT littermates and the animals were randomly divided into experimental groups.

Control group animals in the metabolic experiment received an oral administration of water. L-menthol (Sigma-Aldrich/Merck) was orally administered to study group at doses of 8, 10, 15 and 20 mg/kg. L-menthol was orally administered at a volume of 0.2 mL per 30 g. L-menthol was dissolved in water (Ehrlich *et al.*, 2016).

Eight Wfs1KO mice and eight WT mice were used in the study with 8 mg/kg L-menthol dose. Twelve Wfs1KO and twelve WT mice were used in the menthol study with 10, 15 and 20 mg/kg doses. There were five Wfs1KO mice and eight WT mice in the control group. For adaption, mice were kept alone in cages for one week before metabolic measurement. After adaption period, the mice were studied in the metabolic cages (TSE Phenomaster). Basal data was measured during the first two days in metabolic cages. The basal data of second day was used in the analysis. Within the next four days, one of the following menthol dose 8, 10, 15 or 20 mg/kg was given orally, once per day. Metabolic effect was measured 24 hours after oral menthol administration. For the next four days after 2 days of basal data measurements, 0.2 mL of water was orally administrated to the control group once per day. Rectal temperature ($^{o}$C) was measured 1 and 2 hours after oral menthol administration. Food (g), drink (mL), average $O_2$ consumption (mL/h/kg), average $CO_2$ production (mL/h/kg) and

average heat production (kcal/h/kg) data were collected. The metabolic data of mice were analyzed 3, 7, 12 and 14 hours after oral administration of menthol. Metabolic cages automatically measured and software calculated energy consumption H [kcal/kg/h], taking into account 100% the weight of the mouse (Ehrlich *et al.*, 2016).

## 4.9. High fat diet experiment (Paper IV)

For the high fat diet experiment 15 wild-type (WT) and 16 *Wfs1* heterozygous (Wfs1HZ) mice at the age of 2-3 months were used. Mice were assigned to either low fat (10% fat in kcal; D12450B, Research Diets Inc., USA) or high fat (60% fat in kcal; D12492, Research Diets Inc., USA) diet. The low fat diet (LFD) group had 7 WT and 8 Wfs1HZ mice and the high fat (HFD) group 8 WT and 8 Wfs1HZ mice. The body weight, feed intake and blood glucose were measured weekly. Each week animals were given equal amount of food pellets in grams. The given food was weighed before giving it to the animals and the remaining pellets were weighed before the start of the glucose tolerance test (GTT). The animals had *ad libitum* access to food and water until the start of the GTT. The high fat diet experiment lasted 20 weeks. At the end of the feeding experiment mice were sacrificed with decapitation to collect blood and tissue samples (Ivask *et al.*, 2021).

## 4.10. Glucose tolerance test (Paper IV)

Glucose tolerance test (GTT) in paper IV was carried out as recommended by Mouse Metabolic Phenotyping Center Consortium (Ayala *et al.*, 2010) and the International Mouse Phenotyping Consortium (https://www.mousephenotype.org/impress/protocol/87/12). Blood glucose was measured weekly from the tail tip of 16 h-fasted mice using Accu-Chek Performa glucometer (Roche Diagnostics). Fasted blood glucose levels were determined before 20% glucose (2 g of glucose/kg body mass) (Sigma-Aldrich/Merck) solution was administered by intra-peritoneal (IP) injection. The blood glucose levels were measured at 30, 60, 120 and 180 minutes after glucose injection. Results are expressed as area under curve (AUC), which was calculated using the GraphPad Prism 6 software (GraphPad Software,Inc.) (Ivask *et al.*, 2021).

## 4.11. Plasma insulin analysis (Paper IV)

The plasma insulin levels were measured at the end of the high fat diet experiment in paper IV. The tubes were washed with heparin sodium (5000 IU/mL, B. Braun Melsungen AG) before blood collection. The tubes with collected blood were centrifuged at 2000 g and 4°C for 10 min. Plasma was transferred to a new

tube and stored at -80°C until insulin measurement with Ultra Sensitive Mouse Insulin ELISA Kit (90080, Crystal Chem Inc.) (Ivask *et al.*, 2021).

## 4.12. Statistical analysis (Paper I-IV)

For RNA-sequencing (paper I and II) data analysis sequencing reads were mapped to the mouse genome (version mm10) using the genomic analysis software LifeScope (Life Technologies/Thermo Fisher Scientific). Data were further analyzed for the differential expression with the Bioconductor software package edgeR (Mccarthy *et al.*, 2012, Robinson *et al.*, 2010) implemented in the statistical software R (http://www.r-project.org/) (Ivask *et al.*, 2016, Ivask *et al.*, 2018).

In paper I RT-qPCR data are presented as mean of $2^{-\Delta Ct}$±SD calculated in relation to the housekeeping gene *Hprt* (table 2). Data for studied genes were analyzed by unpaired t-test using GraphPad Prism 6 software (GraphPad Software Inc.) and a *P* value <0.05 was considered significant. Also, the Pearson correlation coefficient of fold change expression between the RNA-seq and RT-qPCR results was calculated using GraphPad Prism 6 (GraphPad Software Inc.) (Ivask *et al.*, 2018).

In paper II RT-qPCR data are presented as mean of $2^{-\Delta Ct}$±SEM calculated in relation to the housekeeping gene *Hprt* (table 2). Data for studied genes were analyzed by one-way ANOVA and Tukey post-test using GraphPad Prism 5 software (GraphPad Software Inc.) and a *P* value <0.05 was considered significant (Ivask *et al.*, 2016).

Islet and insulin secretion data in paper II are presented as mean ± SEM. Data was analyzed using either one-way or two-way ANOVA, followed by Tukey post-test. A *P* value of <0.05 was considered statistically significant (*P*<0.05). The statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc.) (Ivask *et al.*, 2016).

The results of the metabolic experiments in paper III are expressed as mean ± SEM. Welch t-test was applied for the statistical analysis of collected metabolic data. For survival analysis Kaplan-Meyer estimator was used. *P* value lower than 0.05 (*P*<0.05) was considered statistically significant. Statistical analysis was done with statistical computing program R software (http://www.r-project.org/) (Ehrlich *et al.*, 2016).

In paper IV weight and GTT change results were firstly expressed as area under curve (AUC) and then analyzed with one-way or two-way ANOVA followed by Tukey post-test using the GraphPad Prism 6 software (GraphPad Software Inc.), respectively. A *P* value of <0.05 was considered statistically significant (*P*<0.05) (Ivask *et al.*, 2021).

Islet and insulin secretion data in paper IV are presented as mean ± SD. Data were analyzed using either one-way or two-way ANOVA, followed by Tukey post-test. A *P* value of <0.05 was considered statistically significant (*P*<0.05). The statistical analysis was performed using GraphPad Prism 6 software

(GraphPad Software Inc.). Data from RT-qPCR are presented as mean of $2^{-\Delta Ct} \pm SD$ calculated in relation to the housekeeping gene *Hprt* (table 2). Data for studied genes were analyzed by one-way ANOVA and Tukey post-test using GraphPad Prism 6 software (GraphPad Software Inc.) and a $P$ value $<0.05$ was considered significant (Ivask *et al.*, 2021).

# 5. RESULTS

## 5.1. Transcriptome analysis (Paper I, II)

The number of successfully annotated genes by the Ingenuity Pathway Analysis (IPA, Ingenuity Systems) was 21760 in hippocampus and 21800 in hypothalamus. The pancreatic islets RNA-seq also included the Wfs1HZ islets and therefore the number of successfully annotated genes by IPA was 22613 between Wfs1KO and WT, 22619 between Wfs1KO and Wfs1HZ and 22591 between Wfs1HZ and WT.

The number of differentially expressed genes with FDR under 0.05 (FDR<0.05) was 43 in hypothalamus (table 3) and 311 in hippocampus (table 4). In pancreatic islets it was 20 genes between Wfs1KO and WT islets (table 5) and 13 genes between Wfs1KO and Wfs1HZ (table not shown).

**Table 3.** Differentially expressed genes in hypothalamus sorted by FDR*

| Gene | LogFC | *P* value | FDR | Gene name |
|---|---|---|---|---|
| *Wfs1* | -3.045 | 4.26E-56 | 1.02E-51 | Wolfram syndrome 1 (wolframin) |
| *Cdr1* | 2.349 | 4.45E-28 | 5.35E-24 | cerebellar degeneration related antigen 1 |
| *Npm1* | 2.516 | 3.30E-27 | 2.64E-23 | nucleophosmin (nucleolar phosphoprotein B23, numatrin) |
| *Ppig* | 2.406 | 5.58E-22 | 3.35E-18 | peptidylprolyl isomerase G (cyclophilin G) |
| *Hmgn5* | 2.42 | 2.69E-14 | 2.15E-11 | high-mobility group nucleosome binding domain 5 |
| *Spata9* | 2.093 | 1.57E-13 | 8.57E-11 | spermatogenesis associated 9 |
| *Rn45s* | -2.81 | 1.69E-12 | 6.33E-10 | 45S pre-ribosomal RNA |
| *Avpr1a* | 2.385 | 5.32E-10 | 9.88E-08 | arginine vasopressin receptor 1A |
| *Cyth2* | -2.16 | 2.07E-09 | 3.16E-07 | cytohesin 2 |
| *Slc19a1* | -2.377 | 4.92E-08 | 4.63E-06 | solute carrier family 19 (folate transporter), member 1 |
| *Smok4a* | 3.13 | 3.82E-07 | 2.38E-05 | sperm motility kinase 4A |
| *Rab3d* | -2.846 | 4.83E-07 | 2.90E-05 | RAB3D, member RAS oncogene family |
| *Gfra4* | -3.654 | 2.24E-06 | 1.07E-04 | GDNF family receptor alpha 4 |
| *Tsen15* | 2.283 | 4.80E-06 | 1.98E-04 | TSEN15 tRNA splicing endonuclease subunit |
| *Snhg10* | 2.742 | 1.18E-05 | 4.04E-04 | small nucleolar RNA host gene 10 |
| *Rnd2* | -2.365 | 1.63E-05 | 5.24E-04 | Rho family GTPase 2 |
| *Tlr7* | 2.196 | 1.96E-05 | 6.08E-04 | toll-like receptor 7 |
| *Pard6b* | -2.085 | 6.88E-05 | 1.67E-03 | par-6 family cell polarity regulator beta |
| *Kctd16* | -2.221 | 1.25E-04 | 2.72E-03 | potassium channel tetramerization domain containing 16 |
| *Fgfrl1* | -2.164 | 1.29E-04 | 2.78E-03 | fibroblast growth factor receptor-like 1 |

* Table 3 is adapted from Ivask *et al.* (2018) showing only the first 20 results sorted by FDR.

**Table 4.** Differentially expressed genes in hippocampus sorted by FDR*

| Gene | LogFC | *P* value | FDR | Gene name |
|---|---|---|---|---|
| *Trpm8* | 6.624 | 6.00E-203 | 1.44E-198 | transient receptor potential cation channel, subfamily M, member 8 |
| *Camsap3* | 4.749 | 3.08E-156 | 3.70E-152 | calmodulin regulated spectrin-associated protein family, member 3 |
| *Cyb5r2* | -4.919 | 1.26E-84 | 1.01E-80 | cytochrome b5 reductase 2 |
| *Zfyve27* | -2.540 | 1.79E-61 | 1.07E-57 | zinc finger, FYVE domain containing 27 |
| *Rec8* | -5.044 | 1.08E-59 | 4.33E-56 | REC8 meiotic recombination protein |
| *Srpx2* | 5.145 | 2.53E-57 | 8.68E-54 | sushi-repeat containing protein, X-linked 2 |
| *Gpr179* | 3.076 | 3.13E-57 | 9.40E-54 | G protein-coupled receptor 179 |
| *Olfr979* | 7.224 | 2.77E-45 | 6.04E-42 | olfactory receptor, family 10, subfamily G, member 9 |
| *Tktl2* | 4.696 | 9.78E-42 | 1.81E-38 | transketolase-like 2 |
| *Bhlhe41* | -6.129 | 1.12E-40 | 1.93E-37 | basic helix-loop-helix family, member e41 |
| *Col5a2* | 3.579 | 3.08E-37 | 4.93E-34 | collagen, type V, alpha 2 |
| *Lyn* | -3.629 | 8.04E-37 | 1.21E-33 | LYN proto-oncogene, Src family tyrosine kinase |
| *Gcnt7* | 4.152 | 2.19E-34 | 3.09E-31 | glucosaminyl (N-acetyl) transferase family member 7 |
| *Sprr2f* | 4.971 | 1.25E-33 | 1.66E-30 | small proline-rich protein 2F |
| *Zc3h13* | 2.902 | 3.24E-33 | 4.09E-30 | zinc finger CCCH-type containing 13 |
| *Ccl28* | 2.755 | 3.97E-33 | 4.77E-30 | chemokine (C-C motif) ligand 28 |
| *Wfs1* | -2.841 | 7.11E-33 | 8.13E-30 | Wolfram syndrome 1 (wolframin) |
| *Vmn1r171* | -5.640 | 1.28E-32 | 1.40E-29 | vomeronasal 1 receptor 171 |
| *Cox8c* | 4.075 | 2.44E-32 | 2.55E-29 | cytochrome c oxidase subunit VIIIc |
| *Stra8* | 4.662 | 7.25E-30 | 6.97E-27 | stimulated by retinoic acid 8 |

* Table 4 is adapted from Ivask *et al.* (2018) showing only the first 20 results sorted by FDR.

**Table 5.** Differentially expressed genes in pancreatic islets sorted by FDR*

| Gene | LogFC | *P* value | FDR | Gene name |
|---|---|---|---|---|
| *Wfs1* | -2.663 | 2.18E-12 | 5.24E-08 | Wolfram syndrome 1 (wolframin) |
| *Glipr2* | 3.139 | 1.42E-09 | 1.71E-05 | GLI pathogenesis-related 2 |
| *Trpm5* | -2.422 | 4.05E-09 | 3.25E-05 | transient receptor potential cation channel, subfamily M, member 5 |
| *Gad1* | -2.671 | 9.94E-08 | 5.98E-04 | glutamate decarboxylase 1 (brain, 67kDa) |
| *Spock1* | -1.801 | 8.71E-07 | 4.19E-03 | sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1 |
| *Sprr1a* | 2.623 | 1.36E-06 | 5.44E-03 | small proline-rich protein 1A |
| *Bcat1* | 1.821 | 1.67E-06 | 5.75E-03 | branched chain amino-acid transaminase 1, cytosolic |
| *Csf3* | 2.332 | 2.03E-06 | 6.12E-03 | colony stimulating factor 3 (granulocyte) |
| *Nrxn1* | -1.404 | 5.14E-06 | 1.38E-02 | neurexin 1 |
| *Prss23* | 1.769 | 7.19E-06 | 1.62E-02 | protease, serine, 23 |
| *Aw551984* | -1.551 | 7.40E-06 | 1.62E-02 | expressed sequence AW551984 |
| *Cxcl9* | 3.394 | 8.28E-06 | 1.66E-02 | chemokine (C-X-C motif) ligand 9 |
| *MaoB* | -1.86 | 1.02E-05 | 1.89E-02 | monoamine oxidase B |
| *Kcns3* | 4.122 | 1.17E-05 | 2.02E-02 | potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3 |
| *Zfp36* | 1.709 | 1.71E-05 | 2.74E-02 | ZFP36 ring finger protein |
| *Egr1* | 1.392 | 2.65E-05 | 3.97E-02 | early growth response 1 |
| *ApoE* | 1.712 | 2.91E-05 | 3.97E-02 | apolipoprotein E |
| *Itgb3* | 1.701 | 2.97E-05 | 3.97E-02 | integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) |
| *Ccdc85B* | 1.566 | 3.38E-05 | 4.07E-02 | coiled-coil domain containing 85B |
| *Cnnm1* | -1.767 | 3.38E-05 | 4.07E-02 | cyclin M1 |

* Table 5 is adapted from Ivask *et al.* (2016) showing only Wfs1KO compared to WT differentially expressed genes sorted by FDR.

Ingenuity functional pathway analysis software was used for more general functional annotation of the differential gene sets. Network analysis of the genes with lowest *P* values (filter set to $P<0.05$ after FDR correction) of Wfs1KO compared to WT revealed significant enrichment of "inflammatory response, protein synthesis, cell morphology" network in hypothalamus (score 27, Fig. 1) and "tissue morphology, cellular development, hematological system development and function" network in hippocampus (score 42, Fig. 2) (Ivask *et al.*, 2018). In pancreatic islets, the top associated network was "tissue morphology, endocrine system development and function, molecular transport" (score 33, Fig. 3). Analysis of Wfs1KO compared to Wfs1HZ pancreatic islets RNA-seq results revealed a network associated with "cellular development, cellular growth and proliferation, hepatic system development and function" (score 28, figure not shown) (Ivask *et al.*, 2016).

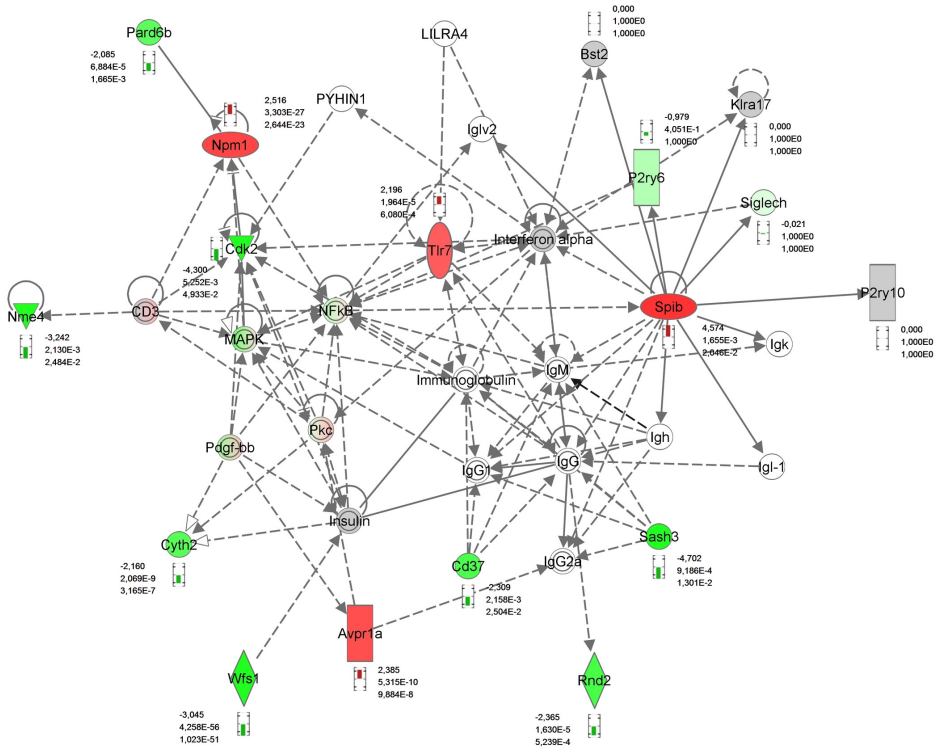


**Figure 1. Top associated functional network of hypothalamus.** Functional annotation revealed that genes with highest expressional changes because of WFS1 deficiency belong to the "inflammatory response, protein synthesis, cell morphology" functional network. Red symbols are upregulated genes, green symbols are downregulated genes, and the numbers reflect the t-value of the statistical comparison with Bayesian moderated t-test (Ivask *et al.*, 2018).

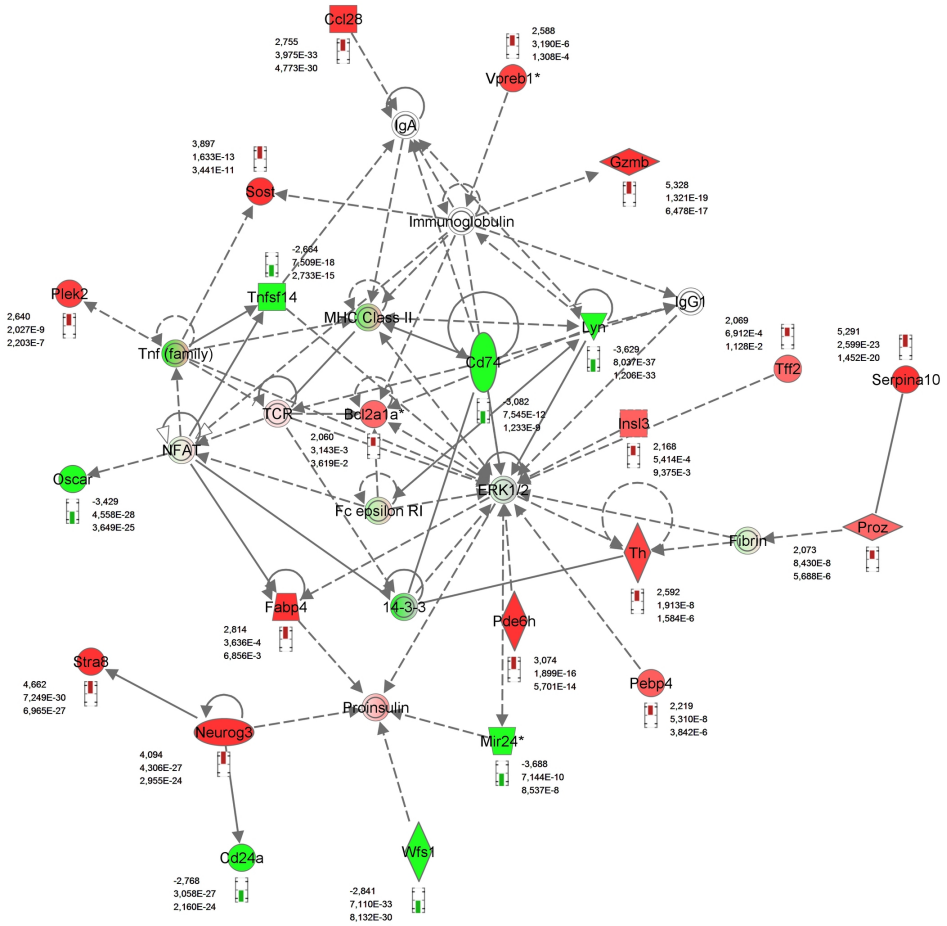


**Figure 2. Top associated functional network of hippocampus.** Functional annotation revealed that differences in genes with highest expressional changes belong to the "tissue morphology, cellular development, hematological system development and function" functional network. Red symbols are upregulated genes, green symbols are downregulated genes, and the numbers reflect the t-value of the statistical comparison with Bayesian moderated t-test (Ivask *et al.*, 2018).

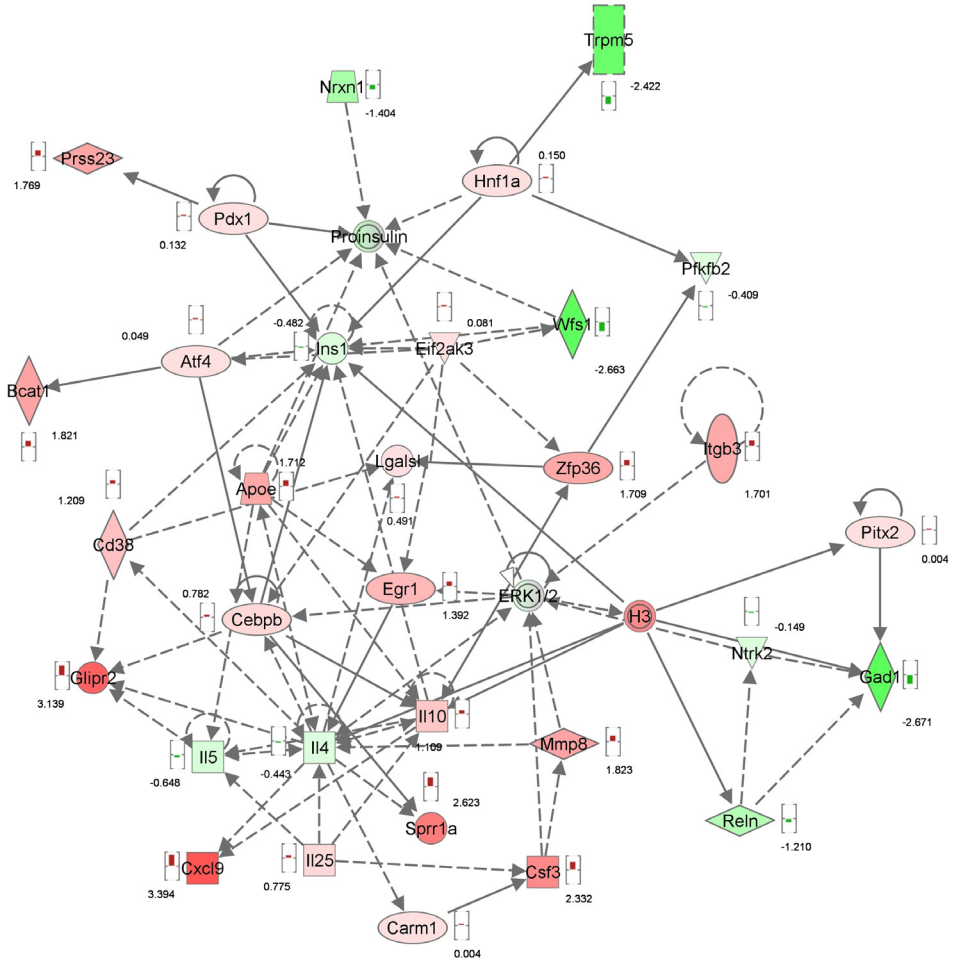


**Figure 3. Top associated functional network of pancreatic islets.** Functional annotation revealed that genes with highest expressional changes because of WFS1 deficiency belong to the "tissue morphology, endocrine system development and function, molecular transport" functional network. Red symbols are upregulated genes, green symbols are downregulated genes, and the numbers reflect the t-value of the statistical comparison with Bayesian moderated t-test (Ivask *et al.*, 2016).

## 5.2. RT-qPCR confirmation of the RNA-sequencing (Paper I, II)

To determine whether similar changes in gene expression as with RNA-seq could be observed with quantitative real-time PCR (RT-qPCR) several genes were selected for analysis. *Wfs1* and *Hspa5* genes were analyzed as a marker genes. The selected TaqMan gene assay for *Wfs1* locates to the exon 7 and exon 8 boundary and therefore also detects the defective gene product.

The selected genes for hypothalamus were *Gfra4*, *Avpr1a* and *Npm1* (Fig. 4). The severely downregulated *Gfra4* could not be determined with RT-qPCR, but the upregulation of *Avpr1a* and *Npm1* in Wfs1KO hypothalamus was confirmed (Ivask *et al.*, 2018).

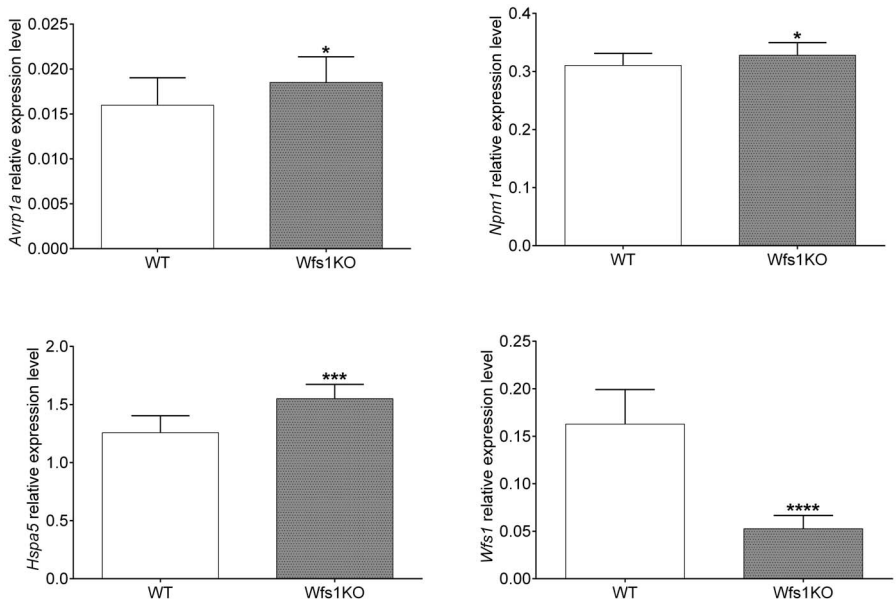


**Figure 4. Hypothalamus confirmatory RT-qPCR results.** *Avpr1a*, *Npm1* and *Hspa5* were significantly upregulated and *Wfs1* downregulated in Wfs1KO. Statistical analysis by unpaired t-test, where **P*<0.05, ***P*<0.01 and ****P*<0.001. Data plotted as mean ± SD, n=5 (Ivask *et al.*, 2018).

For hippocampus, the selected genes were *Trpm8*, *Cyb5r2*, *Ccl28*, *Olfr434* and *Sesn2* (Fig. 5). The severely downregulated *Cyb5r2* and *Olfr434* could not be determined with RT-qPCR in hippocampus samples. The upregulated expression of *Trpm8* and *Sesn2* in hippocampus were statistically confirmed in Wfs1KO mice, but *Ccl28* was not (Ivask *et al.*, 2018).

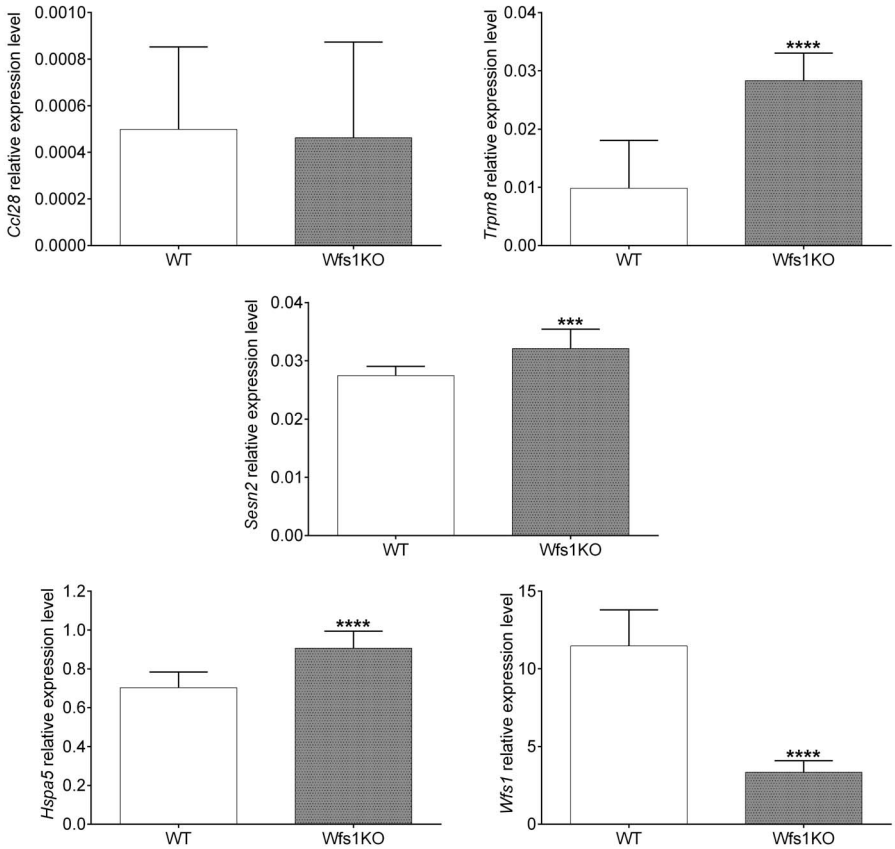


**Figure 5. Hippocampus confirmatory RT-qPCR results.** Upregulation of *Ccl28* in hippocampus was not statistically confirmed. *Trpm8*, *Sesn2* and *Hspa5* were significantly upregulated and *Wfs1* downregulated in Wfs1KO. Statistical analysis by unpaired t-test, where **P*<0.05, ***P*<0.01 and ****P*<0.001. Data plotted as mean ± SD, n=5 (Ivask *et al.*, 2018).

The selected genes for pancreatic islets were *Glipr2, Trpm5, Gad1, MaoB, ApoE* genes (Fig. 6). The upregulation of *ApoE* and *Glipr2* was not statistically confirmed in Wfs1KO pancreatic islets samples with RT-qPCR. The downregulation of *Gad1*, *MaoB* and *Trpm5* was confirmed in Wfs1KO pancreatic islets with RT-qPCR (Ivask *et al.*, 2016).

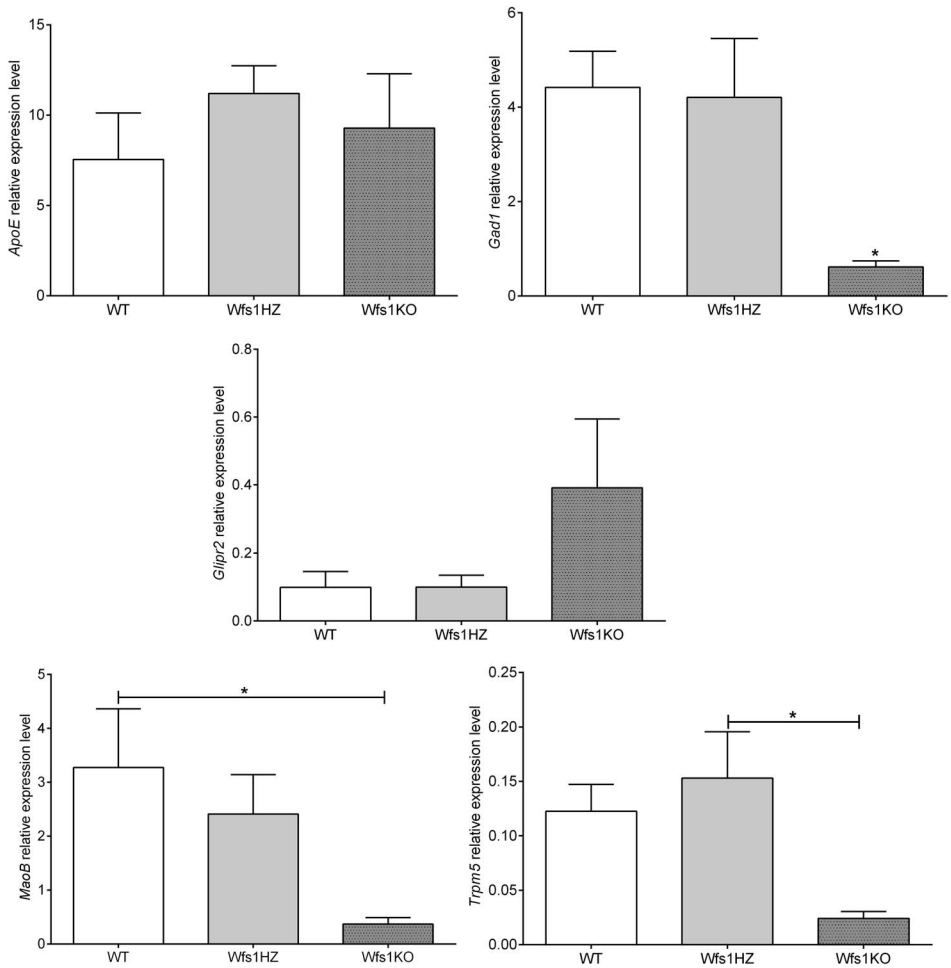


**Figure 6. Pancreatic islets confirmatory RT-qPCR results.** *ApoE* and *Glipr2* were upregulated according to RNA-seq, but RT-qPCR did not confirm it statistically, although there was a trend that *ApoE* and *Glipr2* are upregulated in Wfs1KO islets. The downregulation of *Gad1*, *MaoB*, and *Trpm5* was confirmed in Wfs1KO islets. Statistical analysis by one-way ANOVA followed by Tukey post-test, where **P*<0.05, ***P*<0.01 and ****P*<0.001. Data plotted as mean ± SEM, n=4 (Ivask *et al.*, 2016).

According to RNA-seq *Wfs1* was severely downregulated in all the Wfs1KO tissues and it was also confirmed with RT-qPCR. *Hspa5* was selected as an ER stress marker gene, however, RNA-seq did not show significant changes in the expression of *Hspa5*. The RNA-seq results for *Hspa5* in hippocampus were LogFC = -0.421, *P* value = 7.12E-03, FDR = 6.46E-02, in hypothalamus LogFC = 0.285, *P* value = 5.69E-02, FDR = 2.91E-01 and in pancreatic islets LogFC = 0.858, *P* value = 3.02E-02, FDR = 1.00E+00. *Wfs1* and *Hspa5* expression in pancreatic islets with RT-qPCR are shown in figure 7 (Ivask *et al*., unpublished data).

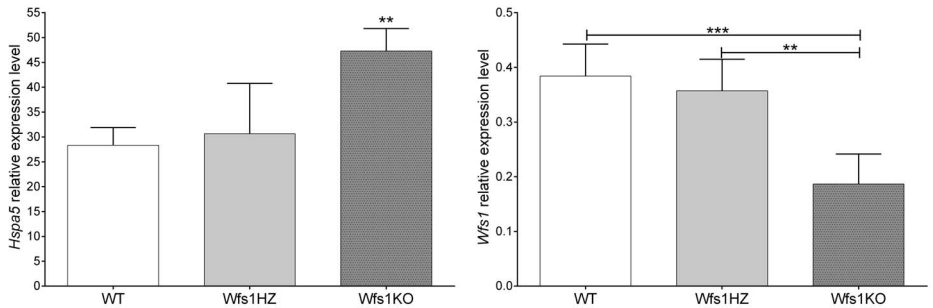


**Figure 7. *Hspa5* and *Wfs1* expression in pancreatic islets with RT-qPCR.** *Hspa5* and *Wfs1* were not upregulated according to RNA-seq, but RT-qPCR showed statistically confirmed upregulation in pancreatic islets Statistical analysis by one-way ANOVA followed by Tukey post-test, where **P*<0.05, ***P*<0.01 and ****P*<0.001. Data plotted as mean ± SEM, n=4 (Ivask *et al*., unpublished data).

## 5.3. Insulin secretion (Paper II)

### 5.3.1. Pancreatic islets and insulin secretion

For *in vitro* insulin secretion assay, the pancreatic islets were isolated and manually handpicked from all three genotype animals. The number of pancreatic islets was manually counted during handpicking. The difference in number of isolated pancreatic islets between the three genotypes was statistically highly significant (WT 303±7.3, Wfs1HZ 176±14 and Wfs1KO 80±7.5, *P*<0.001) (Fig. 8). Wfs1KO animals had remarkably less pancreatic islets than WT or Wfs1HZ animals indicating a genotypic effect.

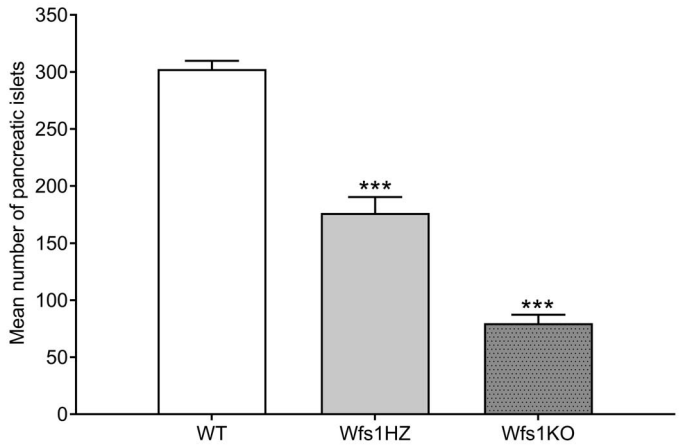


**Figure 8. Comparison of the number of isolated pancreatic islets between three genotypes.** The difference in the number of isolated pancreatic islets per pancreas between genotypes was statistically highly significant (***$P$<0.001) by one-way ANOVA. Data plotted as mean ± SEM, n=6 (Ivask *et al.*, 2016).

The isolated islets were subjected to three different glucose concentration. The basal insulin secretion after incubation in 2 mM glucose (Fig. 9) for Wfs1KO islets was reduced compared to WT and Wfs1HZ islets ($P$<0.001). When islets were stimulated with 10 mM glucose (Fig. 9) solution, the difference in secreted insulin between WT and Wfs1KO was significant ($P$<0.05), the difference between Wfs1HZ and Wfs1KO was highly significant ($P$<0.01). However, after stimulation with 20 mM glucose (Fig. 9) there was no significant difference in stimulation of insulin secretion between the genotypes ($P$>0.05). The response to the potassium channel blocker tolbutamide (Fig. 9) was also significantly impaired in Wfs1KO islets compared to WT ($P$<0.001) and Wfs1HZ ($P$<0.01) islets.

The dose dependent stimulating effect of glucose is seen in all the genotypes, although insulin secretion did not statistically differ after stimulation with 10 mM and 20 mM glucose. The lower normalized insulin amount seen in Wfs1KO mice was primarily due to decreased insulin secretion, because the insulin content in pancreatic islets did not significantly differ between genotypes.

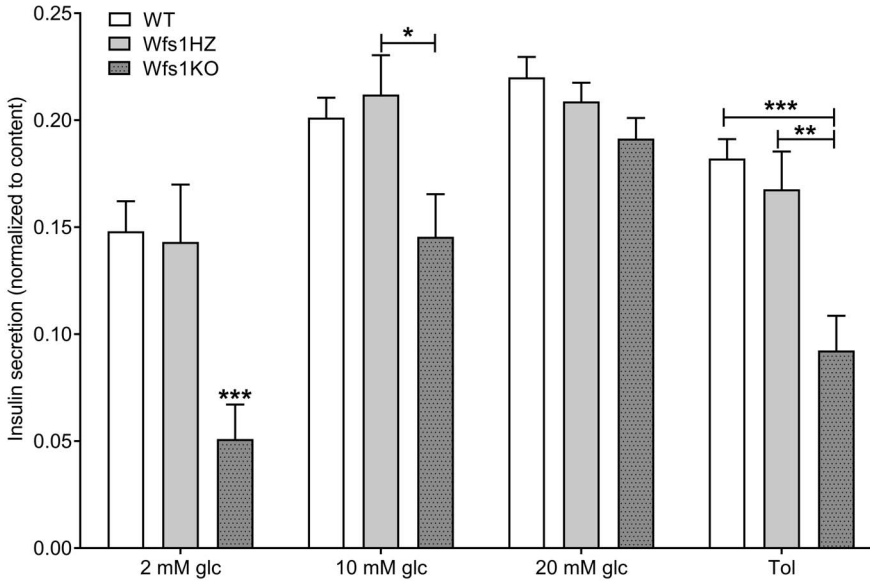


**Figure 9. Insulin secretion from isolated islets.** Insulin secretion from isolated islets of WT, Wfs1HZ and Wfs1KO littermates are compared in response to 2 mmol/L, 10 mmol/L or 20 mmol/L glucose (glc) or 200 µmol/L tolbutamide (Tol) solution and normalized to total insulin content. Insulin secretion from Wfs1KO islets was decreased after incubation in 2 mmol/L and 10 mmol/L glucose and tolbutamide solution, but not after stimulation with 20 mmol/L glucose solution. Statistical analysis by two-way ANOVA, where **P*<0.05, ***P*<0.01 and ****P*<0.001. Data plotted as mean ± SEM, n=6 (Ivask *et al.*, 2016).

### 5.3.2. Proinsulin amount and proinsulin/insulin ratio

Proinsulin was measured from insulin secretion assay samples. The average proinsulin amount per islet (Fig. 10) was not significantly different between the genotypes ($P$>0.05).

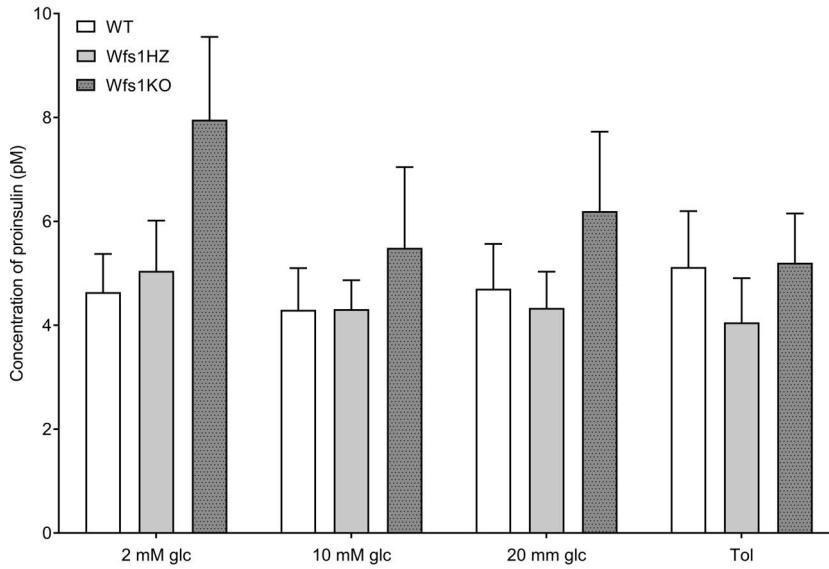


**Figure 10. Comparison of the average amount of proinsulin per islet between the genotypes.** The proinsulin amount per islet was not significantly different between the genotypes ($P$>0.05) after stimulation with 200 µmol/L tolbutamide (Tol) or various glucose (glc) solutions. Statistical analysis by two-way ANOVA, where *$P$<0.05, **$P$<0.01 and ***$P$<0.001. Data plotted as mean ± SEM, n=6 (Ivask *et al.*, 2016).

When islets were incubated in 2 mM glucose solution, there was no significant difference in proinsulin/insulin ratio between WT and Wfs1HZ ($P$>0.05). However, the much higher Wfs1KO proinsulin/insulin ratio was extremely different from ratios of WT and Wfs1HZ genotypes ($P$<0.001) (Fig. 11A). After treating islets with other glucose doses there were no significant difference in proinsulin/insulin ratio between the genotypes ($P$>0.05) (Fig. 11B). However, the ratio of proinsulin/insulin was constantly higher in Wfs1KO mice indicating a larger amount of unprocessed insulin in the islets.

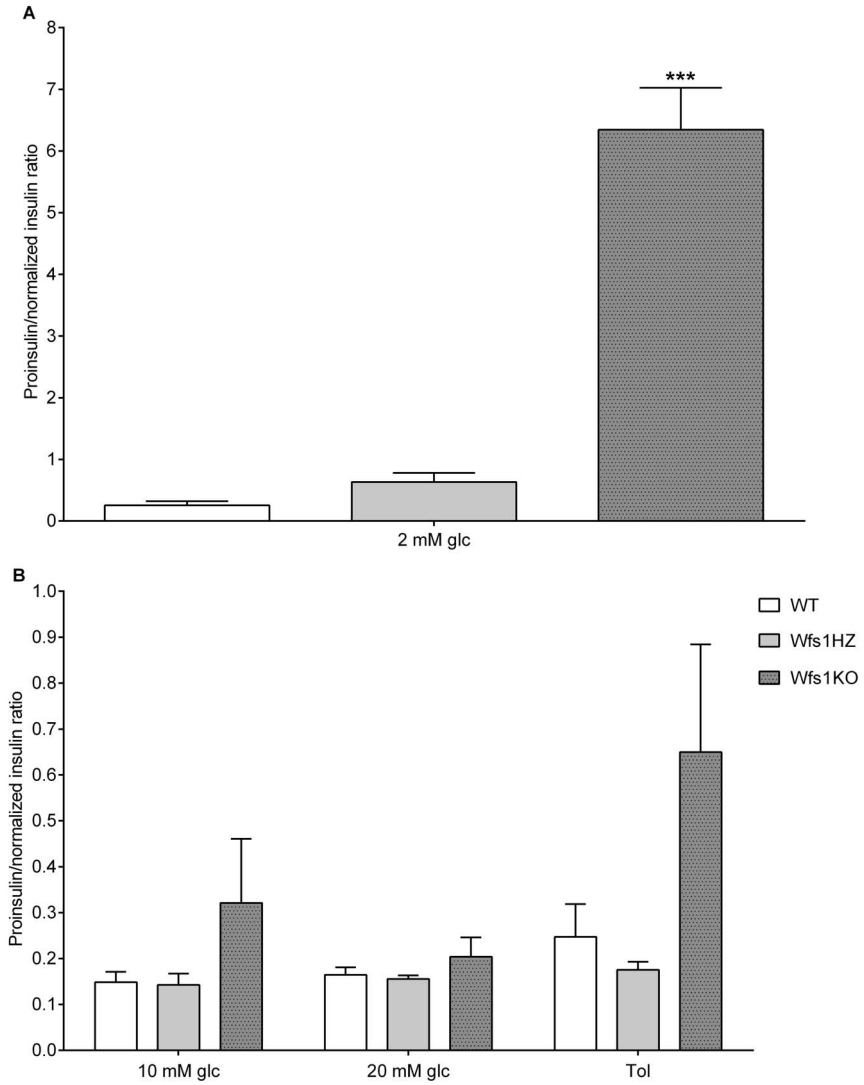


**Figure 11. Proinsulin to insulin ratio.** (A) The higher Wfs1KO proinsulin/insulin ratio was significantly different from WT and Wfs1HZ (***$P$<0.001) at 2 mmol/L glucose (glc). (B) The differences between the genotypes in proinsulin/insulin ratio at higher glucose and tolbutamide (Tol) solutions was not significant ($P$>0.05). Statistical analysis by two-way ANOVA, where *$P$<0.05, **$P$<0.01 and ***$P$<0.001. Data plotted as mean ± SEM, n=6 (Ivask *et al.*, 2016).

## 5.4. Metabolic studies (Paper III)

*Trpm8* (transient receptor potential cation channel, subfamily M, member 8) was the most upregulated gene in the hippocampus of Wfs1KO mice. It has been shown that the cooling agent menthol is an activator for TRPM8 and increases the body core temperature stimulating thermogenesis (Mahieu *et al.*, 2007, Peier *et al.*, 2002). Therefore, in the metabolic studies menthol treatment was included to see its effect on metabolism of Wfs1KO animals with already elevated *Trpm8* expression.

### 5.4.1. Life span

Wfs1KO mice have a shorter life span compared to WT mice ($P$<0.05) (Fig. 12). Average life span of Wfs1KO was 11 months and that of WT mice 15–16 months.

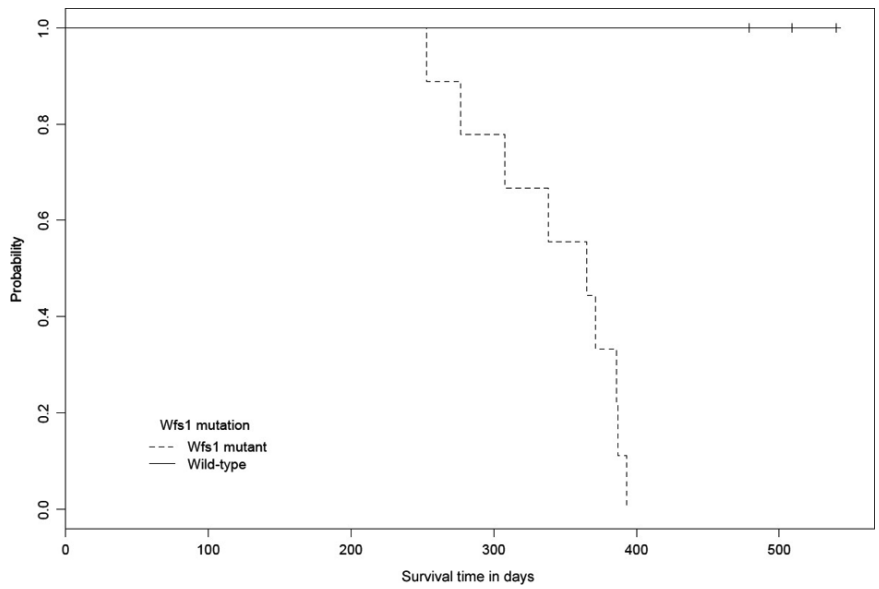


**Figure 12. Comparison of life span between mice of different genotypes.** Wfs1KO mice have a shorter life span compared to WT mice. Wfs1KO n=9, WT n=8 (Ehrlich *et al.*, 2016).

### 5.4.2. Body weight

The general physiological data are presented in the table 6. The body weights were statistically different between genotypes. It was found that Wfs1KO mice (18.38±2.50 g) weigh less compared to WT mice (27.85±3.47 g). Wfs1KO mice did not lose statistically more body weight after menthol treatment compared to WT mice. Wfs1KO mice moderately remained or gained body weight after menthol treatment (0.22±2.44 g) compared to WT mice, which moderately lost body weight after menthol treatment (-0.25±1.33 g).

**Table 6.** Average data of basal and menthol treatment of different metabolic parameters*

| Parameter | WT | Wfs1KO |
|---|---|---|
| Weight (g) | 27.85 ± 3.47*** | 18.38 ± 2.50 |
| Weight change (Δg) | −0.25 ± 1.33 | 0.22 ± 2.44 |
| Food consumption (g) | 2.54 ± 0.94*** | 1.48 ± 1.03 |
| Food consumption (Δg) | 0.24 ± 0.58 | 0.2 ± 0.34 |
| Water consumption (mL) | 2.59 ± 1.58* | 1.76 ± 2.21 |
| Water consumption (ΔmL) | 0.36 ± 0.18 | −0.38 ± 0.23 |
| Body temperature (°C) | 36.86 ± 1.25* | 35.76 ± 2.24 |
| Body temperature change after 1 h menthol administration (Δ°C) | 0.65 ± 1.25 | 0.57 ± 2.24 |
| Body temperature change after 2 h menthol administration (Δ°C) | 0.77 ± 1.25 | 0.68 ± 2.24 |
| $O_2$ consumption (mL/h/kg) | 5435 ± 1108 | 7290 ± 1816*** |
| $CO_2$ production (mL/h/kg) | 5516 ± 1352 | 6827 ± 2177** |
| Heat production (kcal/h/kg) | 27.47 ± 5.78 | 36.07 ± 9.79*** |
| Respiratory coefficient 3 h after menthol administration | 0.89 | 0.89 |
| Respiratory coefficient 7 h after menthol administration | 0.87 | 0.92 |
| Respiratory coefficient 12 h after menthol administration | 0.92 | 0.96 |
| Respiratory coefficient 14 h after menthol administration | 0.94 | 0.97 |

*Table 6 is adapted from Ehrlich *et al.* (2016). Data are expressed as mean values ± SD, where *$P$<0.05, **$P$<0.005 and ***$P$<0.001.

### 5.4.3. Food and water consumption

The food and water intake in Wfs1KO mice (Food = 1.48±1.03 g, Water = 1.75±2.21 mL) was significantly lower compared to WT mice (Food = 2.54± 0.94 g, Water = 2.59±1.58 mL) (table 6). After menthol treatment, Wfs1KO mice did not consume less food or water compared to WT mice.

### 5.4.4. Body temperature

The body temperatures were significantly higher in WT mice (36.86±1.25°C) compared to Wfs1KO mice (35.76±2.24°C) (table 6). Wfs1KO and WT mice body temperature rose 1 and 2 hours after menthol treatment, but the effect was not statistically significant (Fig. 13).

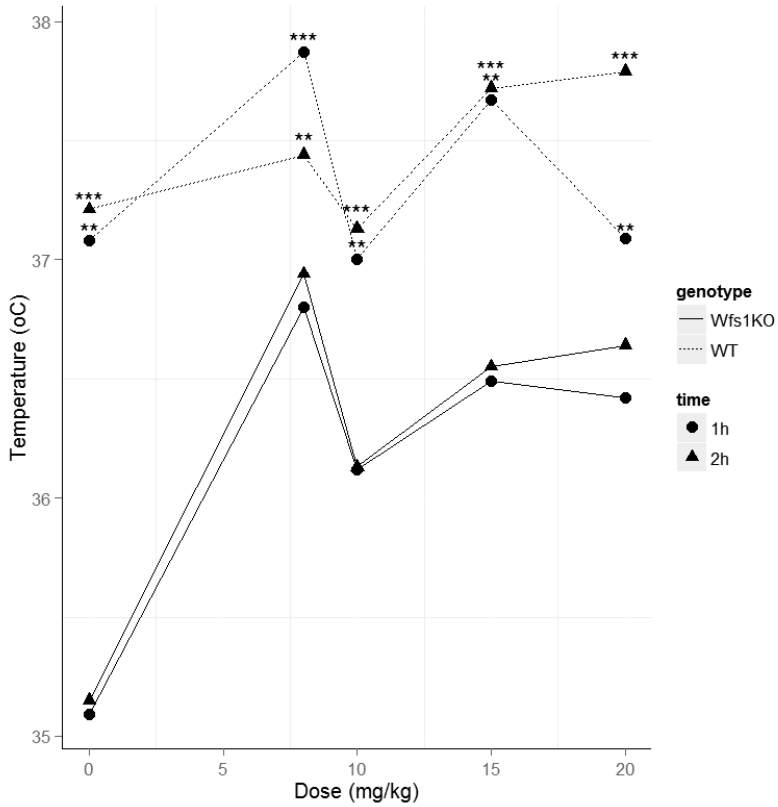


**Figure 13. Effect of menthol treatment on body temperature in Wfs1KO and WT mice.** Different menthol doses did not change the body temperature of Wfs1KO mice and WT mice after 1 and 2 h of oral administration. Wfs1KO – body temperature (°C) of WFS1-deficient mice, WT – body temperature (°C) of wild-type mice. Circle – rectal body temperature 1 h after menthol treatment, triangle – rectal body temperature 2 h after menthol treatment. **P*<0.05, ***P*<0.005, ****P*<0.001 (Ehrlich *et al.*, 2016).

### 5.4.5. Oxygen consumption and carbon dioxide production

Wfs1KO mice ($\Delta O_2$ 7290±1816 mL/h/kg) had significantly higher basal $O_2$ consumption and basal $CO_2$ production ($\Delta CO_2$ 6827±2177 mL/h/kg) compared to WT mice ($\Delta O_2$ 5435±1108 mL/h/kg, $\Delta CO_2$ 5516±1352 mL/h/kg) (Fig. 14). The analysis of average $O_2$ consumption and $CO_2$ production after oral menthol administration showed that the effect on Wfs1KO mice was significantly highest 14 hours after administration compared to WT mice. The strongest effect on Wfs1KO mice was with menthol dose 10 mg/kg (Fig. 14). Menthol treatment increased the average $O_2$ consumption ($\Delta O_2$ 7875±1816 mL/h/kg) and $CO_2$ production ($\Delta CO_2$ 7616±2177 mL/h/kg) of Wfs1KO mice compared to WT mice, whose $O_2$ consumption ($\Delta O_2$ 5329±1108 mL/h/kg) and $CO_2$ production ($\Delta CO_2$ 4988±1352 mL/h/kg) decreased.

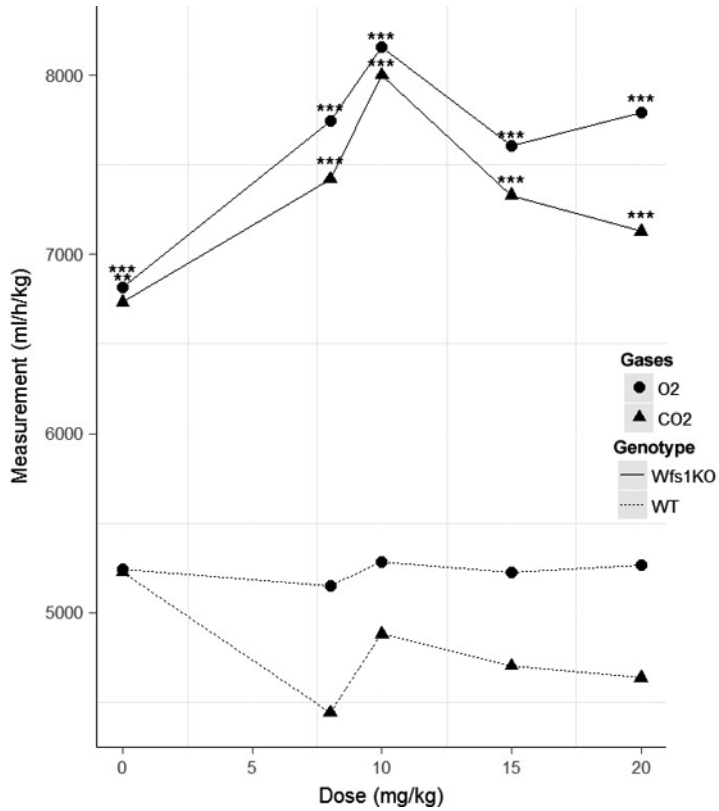


**Figure 14. Oxygen consumption and carbon dioxide production comparison in Wfs1KO and WT mice 12 h after oral menthol administration.** Wfs1KO mice oxygen consumption and carbon dioxide production wass statistically higher after oral menthol administration of doses 8, 10, 15, and 20 mg/kg compared to WT mice. Statistically, highest effect on $O_2$ consumption and $CO_2$ production had menthol dose 10 mg/kg. Wfs1KO – WFS1-deficient mice, WT – wild-type mice, $O_2$ – oxygen consumption (mL/h/kg), $CO_2$ – carbon dioxide production (mL/h/kg). n=8-12 for each group. **P*<0.05, ***P*<0.005, ****P*<0.001 (Ehrlich *et al.*, 2016).

### 5.4.6. Thermogenesis

Wfs1KO mice (H = 36.07±9.79 kcal/h/kg) had significantly higher heat production compared to WT mice (H = 27.47±5.78 kcal/h/kg) (table 6). The average heat production after oral menthol administration showed that the effect on Wfs1KO mice was significantly highest 14 hours after administration compared to WT mice. The strongest effect on Wfs1KO mice was with menthol dose 10 mg/kg (Fig. 15). Fourteen hours after menthol treatment the average heat production of Wfs1KO mice was increased (H = 39.46±9.79 kcal/h/kg) whereas that of WT mice was decreased (H = 26.52±5.78 kcal/h/kg).

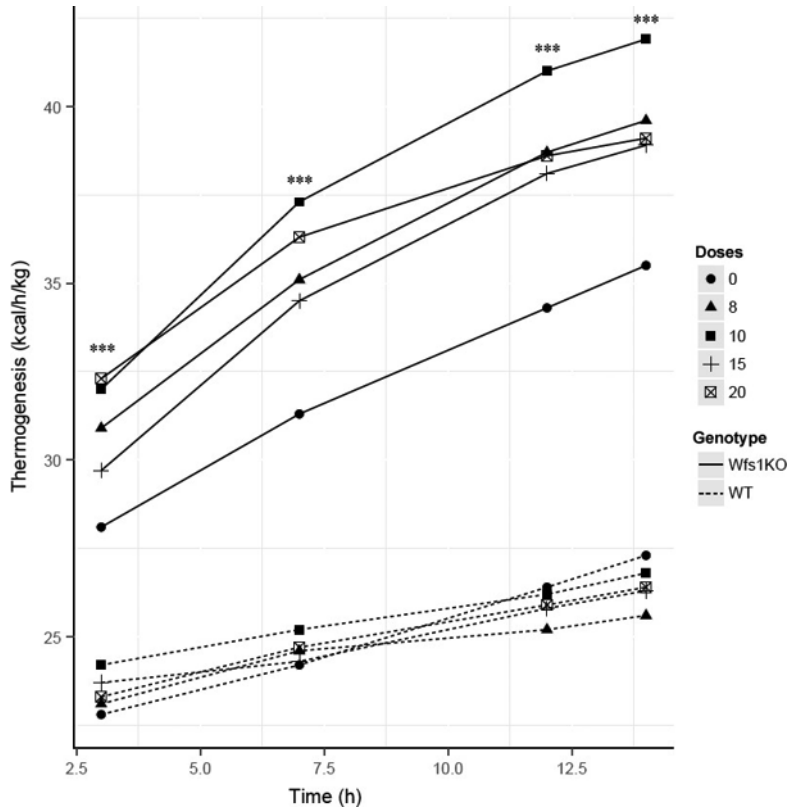


**Figure 15. Menthol activated heat production in Wfs1KO and WT mice.** Wfs1KO heat production was statistically higher after oral menthol administration of doses 8, 10, 15, and 20 mg/kg compared to WT mice. Statistically, highest effect on heat production had menthol dose 10 mg/kg. Wfs1KO – WFS1-deficient mice, WT – wild-type mice, circle – heat production (kcal/h/kg) 3 h after oral menthol administration, triangle – heat production (kcal/h/kg) 7 h after oral menthol administration, square – heat production (kcal/h/kg) 12 h after oral menthol administration, cross – heat production (kcal/h/kg) 14 h after oral menthol administration. n=8-12 for each group. **P*<0.05, ***P*<0.005, ****P*<0.001 (Ehrlich *et al.*, 2016).

## 5.5. The high fat diet influence on Wfs1HZ mice (Paper IV)

### 5.5.1. The high fat diet influence on body weight

In paper IV (Ivask *et al.*, 2021) it was hypothesized that mice with one functional *Wfs1* gene are more susceptible to metabolic disturbances, especially diabetes related changes, caused by high fat diet than mice with two functional alleles. The effects of low and high fat diets (LFD and HFD) were followed for 20 weeks in WT and Wfs1HZ mice. Age-matched mice were divided in different groups based on the diet. The starting body weights (BW) were similar for both genotypes within the LFD or HFD groups. The BW increased more and

continuously in HFD groups during the whole feeding treatment, whereas the BW change became slower and started to stabilize sooner in the LFD groups. The HFD as such resulted in significant weight gains in both WT and Wfs1HZ mice groups. The mean starting weight in the WT-L group was 15.9±3.8 g and at the end of the experiment was 29.0±1.6 g (Fig. 16A), a change of 13.1±3.5 g. In the Wfs1HZ-L group the starting weight was 18.3±1.5 g and at the end 26.3±1.7 g, a change of 8.1±1.7 g. In the WT-H group the starting weight was 22.1±2.3 g and at the end 38.5±3.6 g, a change of 16.4±2.7 g. In the Wfs1HZ-H group the starting weight was 23.1±1.8 g and at the end 40.6±4.1 g, a change of 17.5±3.0 g. The BW increase was smaller in Wfs1HZ than WT mice between LFD groups, but this difference was not seen between HFD groups. In addition, the area under curve (AUC) of weight showed that HFD induced a significant BW gain without any genotype effect (Fig. 16B and Fig. 16C). Thus, these results showed that there was a genotype dependent difference in the BW change due to the LFD, and furthermore that the lower BW increase in Wfs1HZ mice seen in the LFD groups could be prevented by HFD.

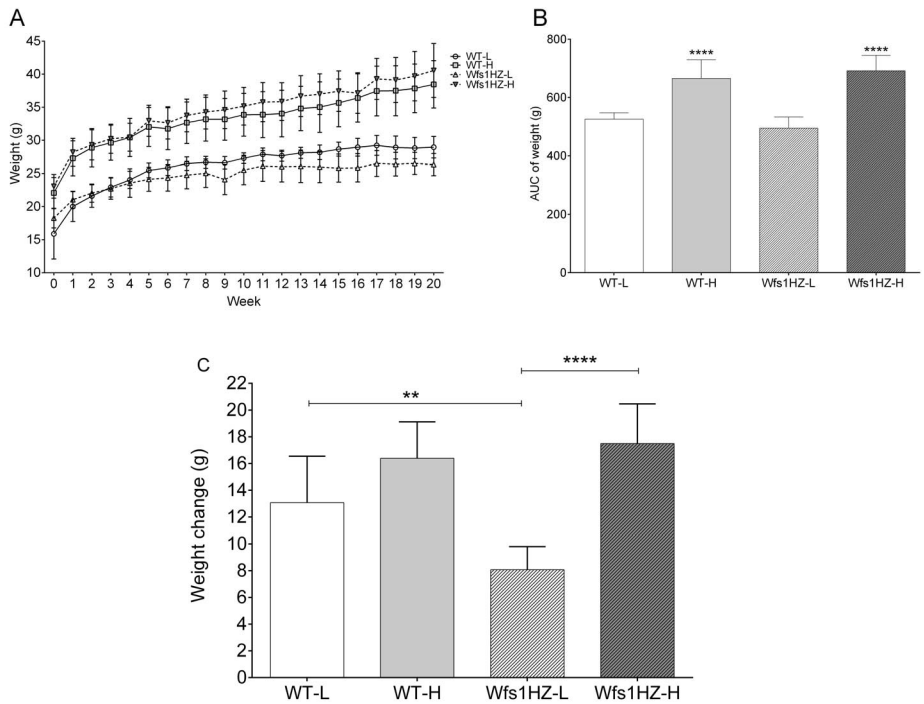


**Figure 16. Weight change during the high fat diet experiment.** A. Weight change from start of the feeding experiment till the end. B. AUC of weight change. C. Mean weight change in grams. Data is displayed as mean ± SD and analyzed with one-way ANOVA followed by Tukey's post-test, where ****$P$<0.0001, n=8 (n=7 for the WT-L group) (Ivask *et al.*, 2021).

### 5.5.2. Glucose tolerance test

The 20 week high fat diet induced changes in the GTT profile in both WT and Wfs1HZ mice groups. At the beginning of the experiment (week 0) the GTT levels did not differ between the groups at different time points (Fig. 17A).

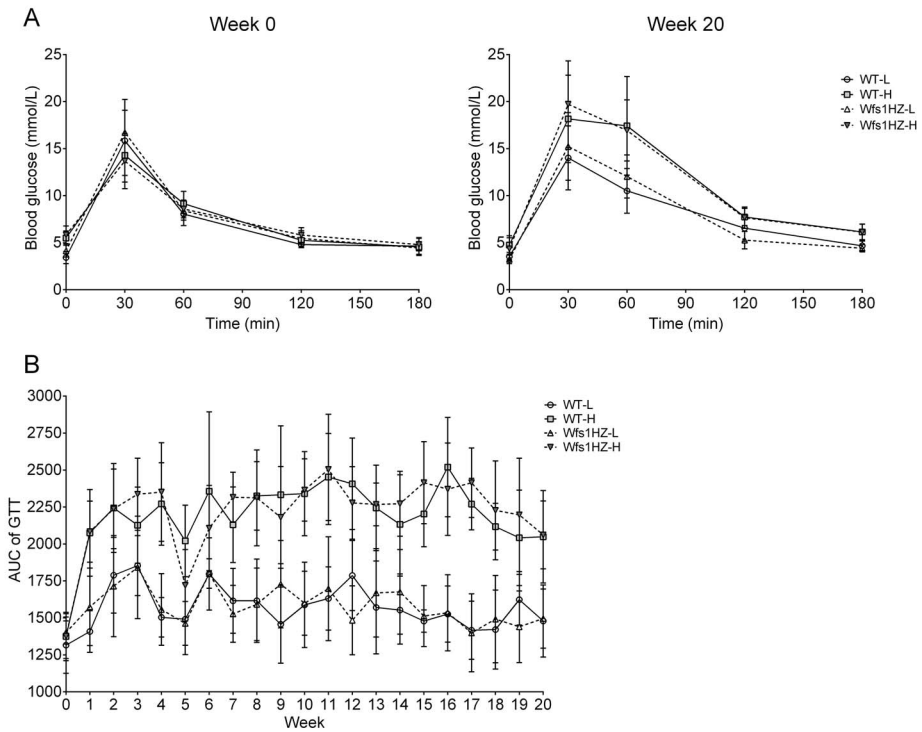


**Figure 17. GTT change over 20 week period.** A. GTT curve at the start of the feeding experiment compared to the curve at the end of the experiment. B. AUC of GTT change. Data is displayed as mean ± SD and analyzed with two-way ANOVA followed by Tukey's post-test. n=8 (n=7 for the WT-L group) (Ivask *et al.*, 2021).

At the end of the experiment (week 20) the basal glucose levels did not differ between the groups, but 30-120 min after glucose administration the GTT levels were elevated for the HFD groups (Fig. 17A). The GTT change during the experiment (Fig. 17B) was only due to the effect of the diet, not the genotype.

### 5.5.3. Insulin secretion following high fat diet

The plasma insulin level was measured in the end of the high fat feeding experiment. The mean plasma insulin level in the WT-L group was 89.23±21.95 ng/mL, in Wfs1HZ-L group 86.89±27.60 ng/mL, in WT-H 87.48±9.10 ng/mL and in Wfs1HZ-H 104.4±30.78 ng/mL (Fig. 18). These differences were not statistically significant.

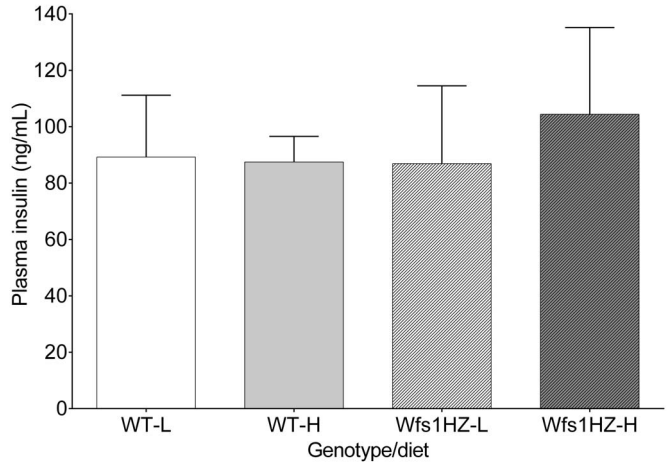


**Figure 18. Plasma insulin levels.** One-way ANOVA with Tukey's post-test, n=8 (n=7 for the WT-L group) (Ivask *et al.*, 2021).

The number of pancreatic islets was manually counted during handpicking. The difference in number of isolated pancreatic islets between groups was not statistically significant (WT-L 233.6±39.7, Wfs1HZ-L 215.8±60.2, WT-H 206.8± 55.5 and Wfs1HZ-H 207.9±47.2) (Fig. 19). Thus, neither the diet nor genotype influenced the number of islets.

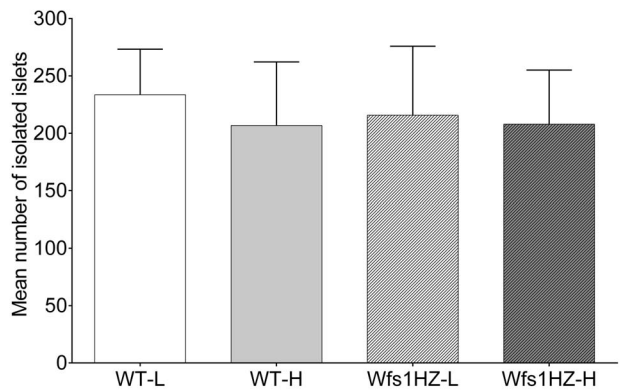


**Figure 19. Mean number of isolated pancreatic islets per group.** The difference in the number of isolated pancreatic islets per group was not statistically different. Analyzed with one-way ANOVA followed by Tukey's post-test. Data plotted as mean ± SD, n=8 (n=7 for the WT-L group) (Ivask *et al.*, 2021).

To reduce the effect of variation in islet size on insulin secretion, the secreted amount of insulin was normalized to the content of insulin (secreted insulin divided by insulin content) (Fig. 20). The normalized insulin ratio was slightly

elevated in Wfs1HZ compared to WT. When treated with 2 mM and 20 mM glucose solution the normalized insulin secretion ratio of Wfs1HZ-H was statistically different from WT-H. When stimulated with 10 mM glucose, Wfs1HZ-H normalized insulin secretion ratio was statistically higher from Wfs1HZ-L group, but there was no difference compared to WT-H. The HFD feeding did not change the normalized insulin secretion in WT mice, whereas the genotype or diet associated increases were found for Wfs1HZ.

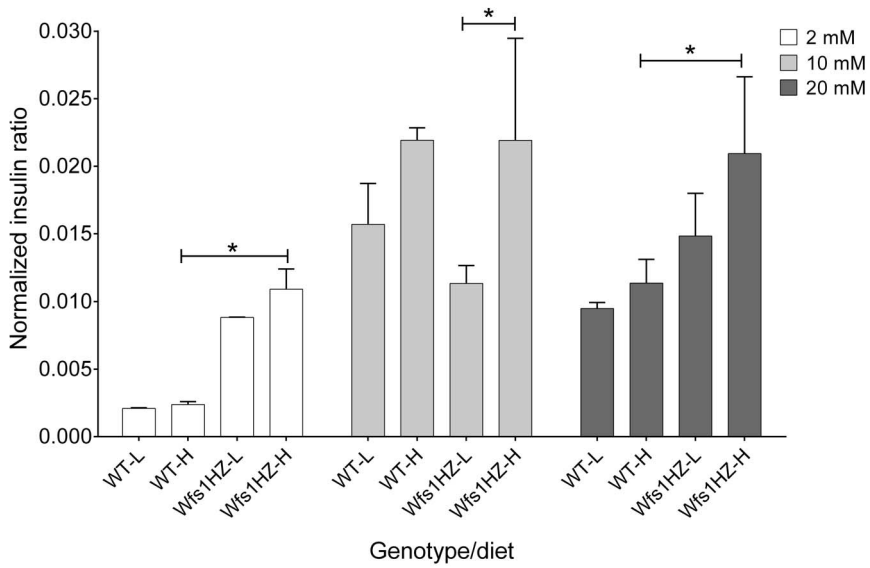


**Figure 20. Normalized insulin ratio of isolated islets.** Insulin secretion from isolated islets was normalized to total insulin content in response to 2 mM, 10 mM or 20 mM glucose solution. Analyzed with two-way ANOVA followed by Tukey's post-test, where *$P$<0.05. Data plotted as mean ± SD, n=8 (n=7 for the WT-L group) (Ivask *et al.*, 2021).

### 5.5.4. ER stress genes expression following high fat diet

The expression of ER stress related genes (*Wfs1*, *Hspa5*, *Atf6α*, *Chop*, *Ire1α*, *Xbp1*, *Atf4*, *Bcl2*, *Jnk* and *Casp3*) were changed when measured with RT-qPCR from liver, kidneys, heart and pancreatic islets. In liver (Fig. 21) the expression of *Ire1α* and *Atf4* were statistically higher in the Wfs1HZ-L group compared to Wfs1HZ-H group. In kidneys (Fig. 22) higher expression of *Wfs1* in the Wfs1HZ-H group compared to Wfs1HZ-L was the only statistically confirmed one. In heart (Fig. 23) the higher expression level of *Chop* was statistically confirmed in the HFD groups and *Wfs1* expression level was statistically higher in WT groups. In pancreatic islets (Fig. 24) the higher expression level of *Chop* and *Ire1α* in the Wfs1HZ-H group compared to Wfs1HZ-L were statistically confirmed.

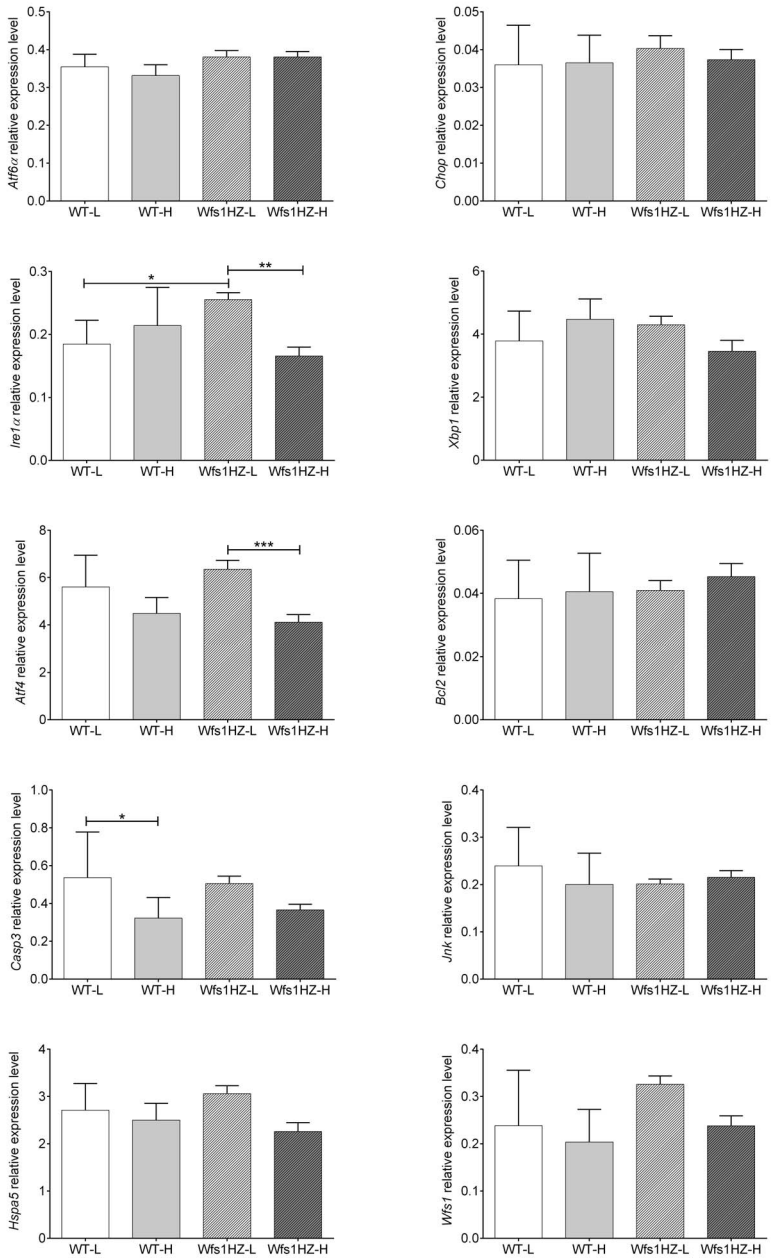


**Figure 21. Gene expression in liver.** There was a trend of the higher expression of *Ire1α*, *Atf4*, *Casp3*, *Hspa5* and *Wfs1* genes in the Wfs1HZ-L group compared to Wfs1HZ-H group. Of these only *Ire1α* and *Atf4* were also statistically confirmed. Statistical analysis by one-way ANOVA followed by Tukey post-test, where **P*<0.05, ***P*<0.01 and ****P*<0.001. Data plotted as mean ± SD, n=8 (Ivask *et al.*, 2021).

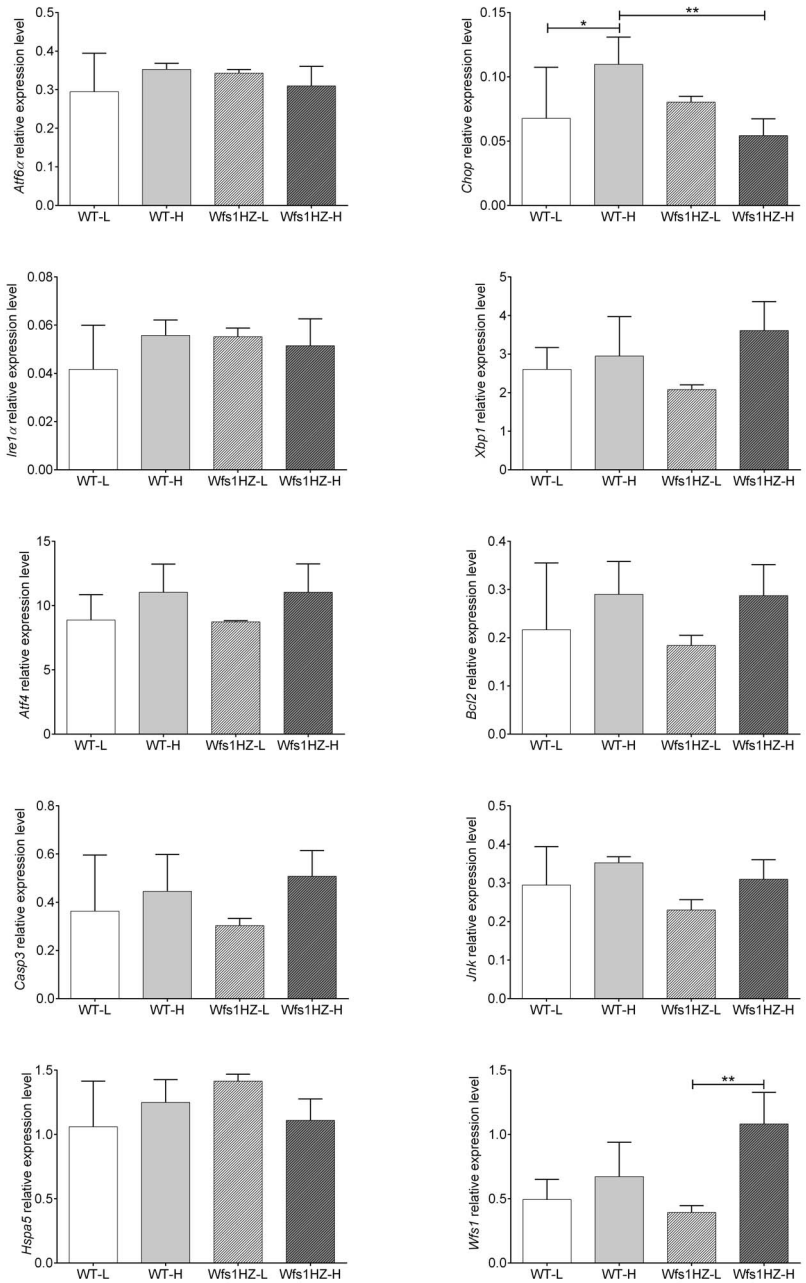


**Figure 22. Gene expression in kidneys.** There was a trend that the expression of *Xbp1*, *Atf4*, *Bcl2*, *Casp3*, *Jnk* and *Wfs1* was higher in both of the HFD groups. However, only the higher expression of *Wfs1* in the Wfs1HZ-H group compared to Wfs1HZ-L was statistically significant. Statistical analysis by one-way ANOVA followed by Tukey post-test, where **P*<0.05, ***P*<0.01 and ****P*<0.001. Data plotted as mean ± SD, n=8 (Ivask *et al.*, 2021).

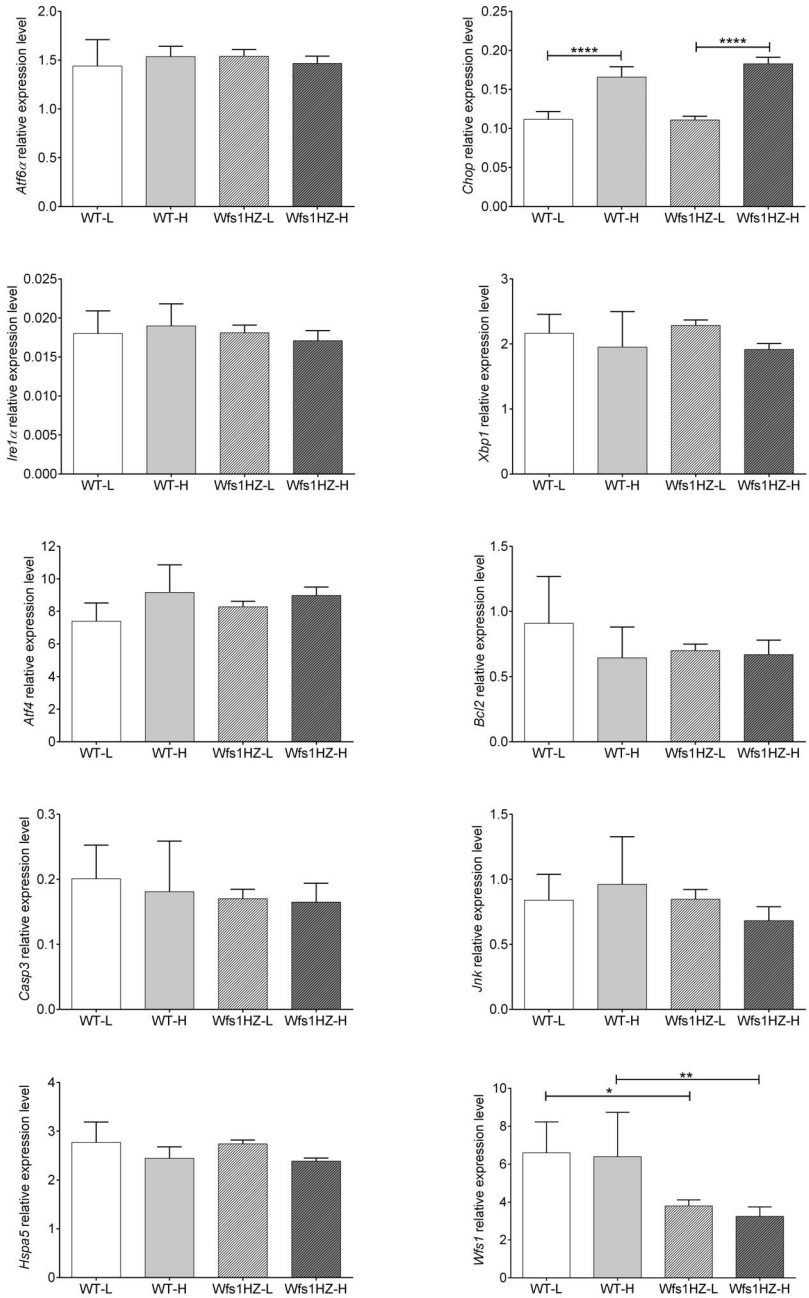


**Figure 23. Gene expression in heart.** The higher expression level of *Chop* in HFD groups was statistically confirmed. In the LFD groups, there was a trend that the expression of *Xbp1* and *Hspa5* was higher. *Wfs1* expression level was statistically higher in WT groups. Statistical analysis by one-way ANOVA followed by Tukey post-test, where **P*<0.05, ***P*<0.01 and ****P*<0.001. Data plotted as mean ± SD, n=8 (Ivask *et al.*, 2021).

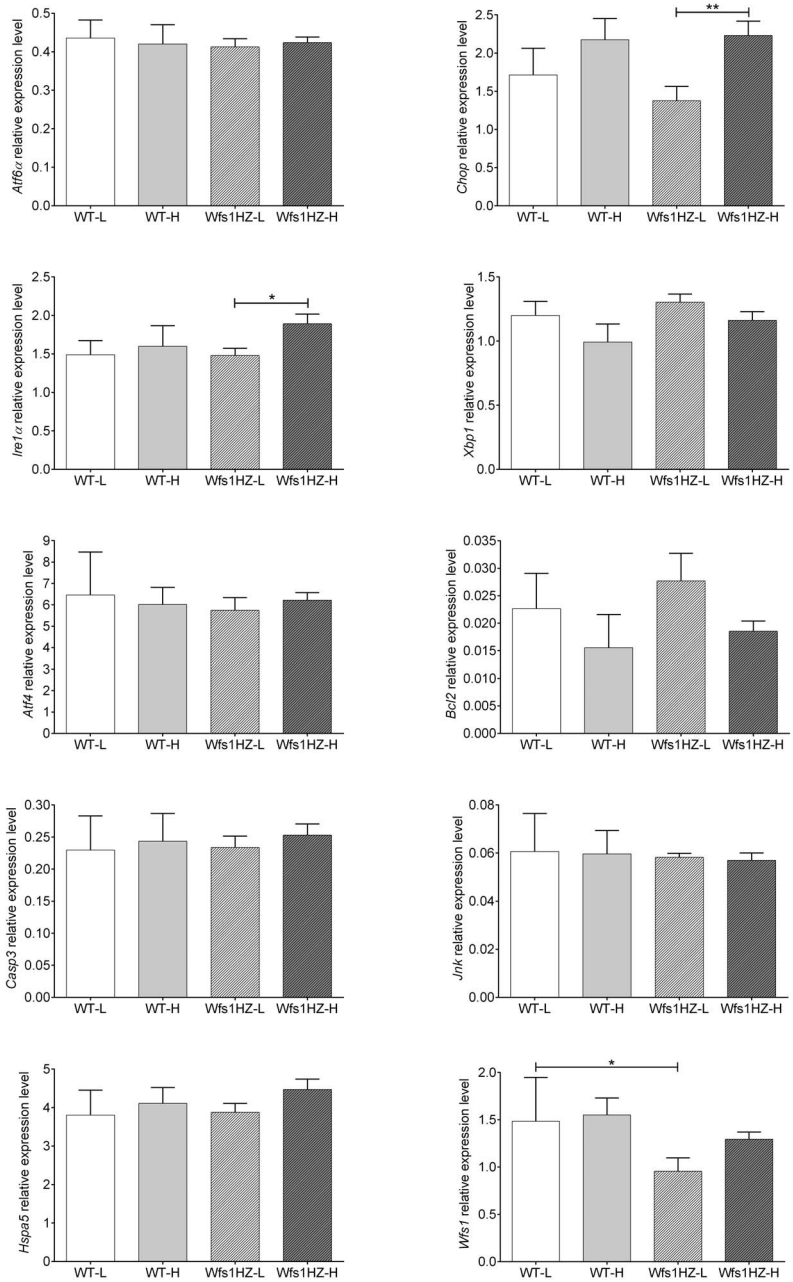


**Figure 24. Gene expression in pancreatic islets.** There was a trend that the expression levels of *Chop*, *Ire1α*, *Hspa5* and *Wfs1* were higher in both HFD groups. *Xbp1* and *Bcl2* expression trended to be higher in LFD groups. The higher expression level of *Chop* and *Ire1α* in the Wfs1HZ-H group compared to Wfs1HZ-L were statistically significant. Statistical analysis by one-way ANOVA followed by Tukey post-test, where **P*<0.05, ***P*<0.01 and ****P*<0.001. Data plotted as mean ± SD, n=8 (Ivask *et al.*, 2021).

# 6. DISCUSSION

## 6.1. Transcriptomic changes influenced by WFS1 deficiency

Wolfram syndrome (WFS) is a heterogeneous disease caused by mutations in the *WFS1* gene, but not much is known what kind of other transcriptomic changes may occur in the organism when WFS1 is dysfunctional. Therefore, the current study first aimed to find possible transcriptomic changes in the hypothalamus, hippocampus, and pancreatic islets of Langerhans in WFS1-deficient mice (Ivask *et al.*, 2016, Ivask *et al.*, 2018). From the RNA-sequencing results, genes associated with metabolism and disturbances common to WFS, like diabetes mellitus and diabetes insipidus, were selected for further analysis. For example, *Avpr1a* (arginine vasopressin receptor 1A) was one of the upregulated genes in the Wfs1KO hypothalamus (table 3, Fig. 4). *Avpr1b* (arginine vasopressin receptor 1B) was significantly upregulated in Wfs1KO hippocampus. According to the RNA-seq results their ligand, neuropeptide arginine vasopressin (*Avp*) gene was downregulated in both tissues, although the FDR values did not confirm the significance (Ivask *et al.*, 2018).

These findings further confirm the underlying molecular changes causing the WFS symptoms, because AVP is a key factor in the pathogenesis of diabetes insipidus and the latter is a characteristic symptom of WFS. The antidiuretic hormone is a neurohypophyseal peptide as it is produced by the hypothalamus and stored in the pituitary gland (Christ-Crain and Gaisl, 2021, Robertson, 2016). AVP has three receptor subtypes: V1a (AVPR1A), V1b (AVPR1B), and V2 receptors. The receptor subtype V2 is mostly expressed in kidneys as it is involved in the diuresis regulation. The subtypes AVPR1A and AVPR1B are broadly expressed in the central nervous system, especially hypothalamus and hippocampus (Jard *et al.*, 1987, Hirasawa *et al.*, 1994, Lolait *et al.*, 1995, Thibonnier *et al.*, 2002, Vaccari *et al.*, 1998). Therefore, AVP influences in addition to neuroendocrine regulation also behavior. There are studies demonstrating the connection between these receptor subtypes and stress-related disorders and behavior, for example, anxiety disorders and depression (Egashira *et al.*, 2009, Landgraf, 2006).

It has been shown that *Avpr1a* and *Avpr1b* knock-out mice have abnormal social behavior. For example, *Avpr1a* knock-out mice have reduced anxiety-like behavior and disturbed spatial learning compared to WT mice (Egashira *et al.*, 2009). Contrarily, *Avpr1a* overexpression increases anxiety-related behavior (Bielsky *et al.*, 2005a). It has been shown that the hypothalamic-pituitary-adrenal (HPA) axis activity is reduced in *Avpr1b* knock-out mice (Tanoue *et al.*, 2004) and they have significantly reduced aggressive behavior as well (Wersinger *et al.*, 2002, Wersinger *et al.*, 2007). Consequently, the increased AVP expression in the hypothalamus is involved in regulation of anxiety-related behavior (Landgraf, 2006), although, the anxiogenic effect of the activation of *Avpr1a* is shown only in males (Bielsky *et al.*, 2005b).

The influence of AVP and its receptors in WFS is not well studied. However, it has been shown that WFS1-deficient mice have elevated anxiety level in stressful environments, as they are less curious and active. Administration of benzodiazepines eliminates the stress-caused vocalization distinctive to Wfs1KO mice and significantly increases their motor activity (Luuk *et al.*, 2008, Luuk *et al.*, 2009). It has also been shown that their dopaminergic system function is decreased (Visnapuu *et al.*, 2013a). As *Avpr1a* in hypothalamus (table 3) and *Avpr1b* in hippocampus were upregulated in paper I (Ivask *et al.*, 2018), it confirms that WFS1 influences the development of diabetes insipidus symptoms in case of WFS. However, the exact mechanism remains understudied. The work by Kurimoto *et al.* (2021) showed that WFS1 is expressed in AVP neurons in the hypothalamus and might influence the AVP system via ER stress activation. The ER stress marker gene *Hspa5* (also known as *BIP*, immunoglobulin binding protein) was upregulated in *Wfs1*-deficient AVP neurons (Kurimoto *et al.*, 2021). *Hspa5* expression was also increased in Wfs1KO mice (Fig. 4-5) supporting the possible interaction (Ivask *et al.*, 2016, Ivask *et al.*, 2018).

Interestingly, *Wfs1* disturbed expression also influences the expression of transient receptor potential channels (TRP channels). *Trpm8* (transient receptor potential cation channel, subfamily M, member 8) was the most upregulated gene in the hippocampus of Wfs1KO mice (table 4, Fig. 5). In Wfs1KO pancreatic islets one of the most significantly downregulated genes was melastatin-related transient receptor potential subfamily member 5 (*Trpm5*) (table 5, Fig. 6) (Ivask *et al.*, 2016, Ivask *et al.*, 2018).

The TRP channels family includes several subfamilies based on the homology of their amino acids and structures (Clapham, 2003, Venkatachalam and Montell, 2007). TRP channels are sensory non-selective cation channels located mostly on the plasma membrane of various cell types. TRP channels have been shown to mediate various activities, for example, body temperature regulation and thermal and pain sensation. The activity of different TRP channels is modified by various endogenous and exogenous stimuli, such as temperature, inflammation, osmolality, pH and irritant substances (Morelli *et al.*, 2013, Nilius *et al.*, 2007).

For example, TRPM8 channels are activated by cold (8-28°C) and chemical stimuli, which may induce cold sensation, like menthol (Nilius *et al.*, 2007). TRPM5 regulates $Ca^{2+}$ homeostasis in the pancreatic islets influencing glucose-stimulated insulin secretion (Brixel *et al.*, 2010, Colsoul *et al.*, 2010). TRPM5 composes a functional calcium-activated nonselective cation channel controlling primarily $Na^{+}$ and $K^{+}$ ions. Its activation causes membrane depolarization downstream of the closure of $K_{ATP}$ channels (Brixel *et al.*, 2010, Prawitt *et al.*, 2003), what in turn may be a crucial for activation of voltage-dependent calcium channels in glucose stimulated insulin secretion (Brixel *et al.*, 2010, Henquin *et al.*, 2009). Colsoul *et al.* (2010) showed that the *Trpm5* mutant islets lack fast oscillations, while maintaining the slow ones. *Trpm5* knock-out mice displayed impaired glucose tolerance because of prolonged elevation of blood

glucose levels (Brixel *et al.*, 2010, Colsoul *et al.*, 2010). Brixel *et al.* (2010) hypothesize that TRPM5 may also have an additional role in the vesicle-membrane fusion process as *Trpm5* mutant islets had decreased insulin secretion regardless of arginine stimulation (Brixel *et al.*, 2010). The suggested hypothesis would support the mechanism of interaction between TRPM5 and WFS1. The functional annotation of Wfs1KO pancreatic islets RNA-seq results also indicated that WFS1 and TRPM5 might be linked over proinsulin processing (Fig. 3) and/or $Ca^{2+}$ signaling (Ivask *et al.*, 2016), because Wfs1KO islets have impaired $Ca^{2+}$ signaling (Ishihara *et al.*, 2004).

RNA-seq of WFS1-deficient pancreatic islets showed that monoamine oxidase B (*MaoB*) was slightly downregulated in Wfs1KO pancreatic islets (table 5, Fig. 6) (Ivask *et al.*, 2016). Reduced monoamine oxidase activity in pancreas has been associated with diabetes. As a mitochondrial enzyme, MAO localizes to the mitochondrial outer membrane, but is found to co-localize also with insulin secretory granules in pancreatic β-cells (Adeghate and Parvez, 2004, Huang *et al.*, 2005). In the study by Ganic *et al.* (2015) it was shown that *MaoB* expression was significantly reduced in type 2 diabetic β-cells contributing to β-cell dysfunction and glucose stimulated insulin secretion disturbances. They hypothesized that loss of monoamine metabolizing enzymes may result in an excess of inhibitory monoamines causing alterations in insulin release particularly during phases of hyperglycaemia. They also suggest that due to reduced MAO activity type 2 diabetic patients experiencing stress and anxiety may be more affected by the fluctuations of neurotransmitter signals inhibiting insulin release promoting hyperglycaemia (Ganic *et al.*, 2015). Unfortunately, the roles of monoamines and monoamine oxidase in the pancreatic islets and in the development of diabetic symptoms are understudied in WFS1-deficient mice. So far, there is evidence showing alterations in serotonergic and dopaminergic systems in the central nervous system (Visnapuu *et al.*, 2013a, Visnapuu *et al.*, 2013b).

In conclusion, the transcriptomic changes influenced by WFS1 deficiency in the hypothalamus, hippocampus and pancreatic islets are evident. The changes in these brain areas result in different endocrine, neurodegenerative and behavioral phenotypes in WFS1-deficient mice as well as Wolfram syndrome patients. Studying the WFS1 function results in new knowledge about Wolfram syndrome, contributing in finding ways to treat the comorbid symptoms and raise the life quality of the patients.

## 6.2. Insulin secretion

WFS1-deficient mice and rats have disturbed blood glucose regulation and glucose stimulated insulin secretion (Fig. 9) (Ivask *et al.*, 2016, Luuk *et al.*, 2009, Noormets *et al.*, 2011, Plaas *et al.*, 2017, Terasmaa *et al.*, 2011). The significantly reduced number of pancreatic islets in WFS1-deficient mice as one of the causes for impaired insulin metabolism has been shown in previous

(Ishihara *et al.*, 2004, Riggs *et al.*, 2005) and current paper II (Fig. 8) (Ivask *et al.*, 2016). The immunohistochemistry analysis of the pancreas of the same WFS1-deficient mouse model used in current thesis demonstrated also their smaller size (Terasmaa *et al.*, 2011). Similarly, the β-cell mass is decreased in Wfs1KO rats and by 14 months of age the pancreatic islets seem to be visually absent in the Wfs1KO rats (Plaas *et al.*, 2017).

In paper II (Ivask *et al.*, 2016) it was shown that WFS1-deficient pancreatic islets secreted less insulin following glucose stimulation (Fig. 9). The study had some inconsistencies compared to the hypothesis of Wfs1KO islets secreting less insulin regardless of the glucose concentration as there was no statistically significant difference after stimulation with 20 mmol/L glucose between the genotypes. The increase in insulin secreted after stimulation with 10 mmol/L compared to 20 mmol/L glucose was greater for Wfs1KO islets while the amount of secreted insulin stayed more or less at the same level for WT and Wfs1HZ islets, which was not expected and could not be explained (Ivask *et al.*, 2016). The findings are still in accordance with previous studies (Ishihara *et al.*, 2004, Riggs *et al.*, 2005), although Ishihara *et al.* (2004) did not observe a difference in insulin secretion after incubation with low glucose. This variation could be due to differences in *Wfs1* mouse models as WFS1-deficient mice in paper II (Ivask *et al.*, 2016) had a disrupted exon 8 (Koks *et al.*, 2009) and the other mice used by Ishihara *et al.* (2004) were a full knock-out of exon 2.

Wfs1KO islets in paper II (Ivask *et al.*, 2016) exhibited reduced insulin secretion following stimulation with sulfonylurea tolbutamide (Fig. 9). Tolbutamide causes the β-cells to release insulin independent of glucose metabolism by blocking the ATP-sensitive $K^+$ ($K_{ATP}$) channels inducing membrane depolarization, $Ca^{2+}$ influx and insulin release (Ashcroft and Rorsman, 1989). WFS1 probably affects insulin secretion downstream of the $K_{ATP}$ channel signaling mechanism (Ivask *et al.*, 2016). Several studies have linked WFS1 and ER $Ca^{2+}$ level regulation, including $Ca^{2+}$ leak from the ER and rise of its levels in the cytosol in case of WFS1 deficiency. WFS1-deficient pancreatic β-cells have impaired $Ca^{2+}$ regulation leading to reduced $Ca^{2+}$ stores, what alongside with elevated ER stress may contribute to the increased apoptosis of the β-cells (Ishihara *et al.*, 2004, Osman *et al.*, 2003, Takei *et al.*, 2006). Hara *et al.* (2014) demonstrated that WFS1 regulates $Ca^{2+}$ efflux from the ER and the ER $Ca^{2+}$ depletion with consecutive surge in cytoplasmic $Ca^{2+}$ activates calpain-2 leading to β-cell death. Zatyka *et al.* (2015) showed in WFS1-depleted MIN6 cells that $Ca^{2+}$ and ATP levels were reduced following glucose stimulation. In addition it was demonstrated that sarco(endo)plasmic reticulum ATPase (SERCA) expression was increased in several WFS1-depleted cell lines and primary pancreatic islets indicating that WFS1 is involved in negative regulation of SERCA (Zatyka *et al.*, 2015). $Ca^{2+}$ signaling is also important for ER and mitochondria interaction. The ER-mitochondria communication, what regulates energy metabolism and cell survival, seems to be impaired in case of WFS1 deficiency, leading to metabolic dysfunction and neurodegeneration. WFS1 may affect ER-mitochondria crosstalk via interacting with neuronal calcium

sensor 1 (NCS1) and inositol-1,4,5-trisphosphate receptor (IP3R) promoting $Ca^{2+}$ exchange between the ER and mitochondria (Angebault *et al.*, 2018). Angebault *et al.* (2018) found that NCS1 level was reduced in fibroblast cells from a WFS patient, but overexpressing it restored ER-mitochondria interactions and mitochondrial $Ca^{2+}$ uptake. Similar findings were found also when *Wfs1* was knocked out in rat insulinoma (INS1) cells. Following *Wfs1* knockout the cells had elevated cytosolic $Ca^{2+}$, reduced stimulus-evoked $Ca^{2+}$ signaling, were more susceptible to hyperglycemia and had decreased insulin secretion. The observed effects were reversed with WFS1 or NCS1 overexpression (Nguyen *et al.*, 2020).

Paper II showed that there was no difference in insulin secretion between Wfs1HZ and WT mice (Fig. 9) (Ivask *et al.*, 2016). *In vivo* studies have also shown that Wfs1HZ mice are more similar to WT and do not exhibit lower plasma insulin levels like Wfs1KO mice (Noormets *et al.*, 2011, Terasmaa *et al.*, 2011). The decreased insulin levels in Wfs1KO mice are probably because of defective or reduced insulin release from the pancreatic islets, because the insulin levels inside the pancreatic islets did not remarkably differ between the genotypes (Ivask *et al.*, 2016). WFS1 has been shown to localize besides β-cells also to the secretory granules. Defective WFS1 causes changes in the intragranual pH creating disturbances in proinsulin processing (Hatanaka *et al.*, 2011). Consequently, the level of circulating proinsulin is higher in WFS1-deficient mice (Noormets *et al.*, 2011).

In paper II the amount of proinsulin extracted from the pancreatic islets did not significantly differ between the genotypes, however, the proinsulin/insulin ratio was higher in Wfs1KO (Fig. 10-11) (Ivask *et al.*, 2016). Higher proinsulin/insulin ratio has been associated with type 2 diabetes (Mykkanen *et al.*, 1997) implying that WFS1-deficient mice have a diabetes-like phenotype with problematic conversion of proinsulin to insulin and release of insulin from secretory granules. The findings from Hatanaka *et al.* (2011) and Ivask *et al.* (2016) are further confirmed in the study by Wang *et al.* (2021). Wang *et al.* (2021) showed that WFS1 is needed for transferring vesicular cargo proteins from ER to Golgi complex. The ER luminal C-terminal segment of WFS1 was bound to vesicular cargo proteins, like proinsulin, and in case of C-terminal mutations in the *Wfs1* this interaction was impaired both in *in vitro* and *in vivo*. Also the proinsulin/insulin ratio was higher in case of defective WFS1 (Wang *et al.*, 2021).

Surprisingly, the RNA-seq of WFS1-deficient pancreatic islets did not show down- or upregulation of various ER stress markers (Ivask *et al.*, 2016). However, it has been shown that pancreatic β-cells with non-functional WFS1 are more prone to ER stress and apoptosis induced by unresolved or high ER stress, causing the degeneration and destruction of pancreatic islets in WFS1-deficient mice (Fonseca *et al.*, 2005, Fonseca *et al.*, 2009, Fonseca *et al.*, 2010, Ishihara *et al.*, 2004, Philbrook *et al.*, 2005, Riggs *et al.*, 2005, Yamada *et al.*, 2006). Several studies have demonstrated that disruption of WFS1 elevates the levels of ER stress marker genes from all three UPR pathways (Fonseca *et al.*,

2005, Fonseca *et al.*, 2009, Fonseca *et al.*, 2010, Yamada *et al.*, 2006, Yamaguchi *et al.*, 2004). WFS1 has been shown to regulate ATF6α activity and suppressing ATF6α-mediated activation of the ER stress response (Fonseca *et al.*, 2010). The expressions of HSPA5 (also known as BIP or GRP78), GRP94 (also known as HSP90B1) and spliced XPB1 are increased in the WFS1-deficient pancreatic β-cells (Kakiuchi *et al.*, 2009, Yamada *et al.*, 2006). Although, RNA-seq did not confirm the higher expression of these ER stress markers in Wfs1KO pancreatic islets, the elevated expression of *Hspa5* was confirmed with RT-qPCR method (Fig. 7) (Ivask *et al*., unpublished).

In conclusion, Wfs1KO mice have fewer pancreatic islets and defective insulin secretion contributing to the diabetes-like phenotype. RNA-sequencing of pancreatic islets showed that *Trpm5* is downregulated in WFS1-deficient islets and should be investigated in future WFS1-related studies. The pathways related to tissue morphology, endocrine system development and function, molecular transport network are influenced by WFS1 deficiency in Wfs1KO mice.

## 6.3. Metabolism of WFS1-deficient mice

The RNA-seq of Wfs1KO hippocampus revealed an increased expression of the transient receptor potential melastatin 8 (*Trpm8*) (table 4, Fig. 5) (Ivask *et al.*, 2018). TRPM8 is a $Ca^{2+}$-permeable cold-sensing non-selective cation channel and has a role in the regulation of steady-state $Ca^{2+}$ level in the ER and mitochondria (Bidaux *et al.*, 2018). It has been shown to be expressed in neurons sensing temperature and pain (Nilius and Voets, 2007, Voets *et al.*, 2004) and cooling agents, like menthol and icilin, are its activators (Mahieu *et al.*, 2007, Peier *et al.*, 2002). As studies have shown that TRPM8 has a role in thermoregulation and is activated by menthol, paper III was conducted to see the effects of menthol on the Wfs1KO metabolism (Ehrlich *et al.*, 2016).

In paper III the Wfs1KO mice had significantly lower body weight, however they did not lose statistically more body weight after menthol treatment compared to WT mice (table 6) (Ehrlich *et al.*, 2016). It has been shown that mice getting dietary menthol have higher body temperature and increased activity, but their body weight does not differ when eating regular chow diet with or without menthol (Ma *et al.*, 2012). In general, Wfs1KO mice display growth retardation starting from 2-3 months of age, causing them to weigh less compared to the WT littermates even on regular chow diet and group housing (Koks *et al.*, 2009, Luuk *et al.*, 2009, Noormets *et al.*, 2014).

The metabolic changes and behavior has not been investigated in case of WFS, but because of the neurodegenerative course of the syndrome it is important for better patient care (Strom *et al.*, 1998). Patients with different neurodegenerative diseases, like Alzheimer's, Parkinson's, or Huntington's disease, might also have defective glucose and insulin metabolism and/or abnormal appetite regulation. For example, people with Huntington's disease may lose more weight despite of normal appetite and high calorie consumption

compared to healthy controls, because of possible increased energy expenditure (Cai *et al.*, 2012). WFS patients and Wfs1KO mice also have a shorter life span (Fig. 12) (Ehrlich *et al.*, 2016, Urano, 2016).

In paper III Wfs1KO mice consumed less food and water and had higher basal $O_2$ consumption, $CO_2$ production and heat production, although their body temperature was lower (table 6, Fig. 13–15) (Ehrlich *et al.*, 2016). However, in the study by Noormets *et al.* (2014) the mean heat production did not differ between Wfs1KO and WT mice. The female Wfs1KO mice had higher mean $O_2$ consumption compared to Wfs1KO males, but no such difference was seen in WT mice. The difference from Noormets *et al.* (2014) might be because of the age of the mice used as in paper III the mice were 9-12 months old compared to 2-3 months in the study by Noormets *et al.* (2014).

Following menthol treatment in paper III the food and water consumption of Wfs1KO mice was no longer statistically different from WT mice, but the average $O_2$ consumption and $CO_2$ production increased more in Wfs1KO mice (table 6, Fig. 14) (Ehrlich *et al.*, 2016). Ma *et al.* (2012) demonstrated that long-term dietary menthol treatment had no effect on food intake, but increased $O_2$ consumption in WT mice. As the same effects were not seen in *Trpm8*$^{-/-}$ mice, they concluded that menthol supplementation activated TRPM8 causing elevated resting metabolic rate (Ma *et al.*, 2012). In Wfs1KO mice the higher expression of *Trpm8* might therefore induce increased basal $O_2$ consumption, $CO_2$ production and heat production compared to WT mice and these effects are amplified by menthol administration (Ehrlich *et al.*, 2016), however, further studies are needed to investigate the possible interaction.

In conclusion, Wfs1KO have metabolic disturbances as they have shorter life span, weigh less and the metabolic rate is different from WT littermates. The effect of dietary menthol on metabolic parameters is more pronounced in Wfs1KO mice that might be because of increased expression of *Trpm8*, but further studies are needed to clarify the exact mechanism.

## 6.4. High fat diet influence on Wfs1HZ mice

In paper IV it was hypothesized that *Wfs1* heterozygous animals (Wfs1HZ) are more prone to environmental insults, like high fat diet (HFD), inducing metabolic complications (Ivask *et al*., 2021). Different genome-wide studies have demonstrated that mutations in *WFS1* increase the risk of developing type 2 diabetes (T2D) (Cheurfa *et al.*, 2011, Florez *et al.*, 2008, Franks *et al.*, 2008, Minton *et al.*, 2002, Sandhu *et al.*, 2007, Van Hoek *et al.*, 2008) and metabolic disorders, for instance T2D, have been associated with chronically elevated ER stress contributing to the progression of metabolic syndrome. Chronic over-feeding leads to obesity that is one of the most important risk factors in the T2D pathogenesis. Obesity affects carbohydrate and lipid metabolism and induces glucolipotoxicity causing ER stress (Bhattarai *et al.*, 2020, Fernandes-Da-Silva *et al.*, 2021). Number of studies have shown that WFS1 is important for ER

homeostasis and ER stress regulation. WFS1-deficient pancreatic β-cells are more susceptible to ER stress and unresolved ER stress induced apoptosis (Fonseca *et al.*, 2005, Fonseca *et al.*, 2009, Fonseca *et al.*, 2010, Ishihara *et al.*, 2004, Philbrook *et al.*, 2005, Riggs *et al.*, 2005, Yamada *et al.*, 2006).

In paper IV (Ivask *et al.*, 2021) consuming HFD increased the body weight of Wfs1HZ as well as the body weight of WT mice, indicating the effect of the diet, not the genotype (Fig. 16). Interestingly, HFD evened the body weight differences between WT and Wfs1HZ animals, although the difference in body weight was still present between LFD fed WT and Wfs1HZ mice. Previous studies on the same WFS1-deficient mouse model have shown that the body weight of the Wfs1HZ mice is between WT and Wfs1KO mice, the latter having reduced growth and body weight starting from 2-3 months of age (Koks *et al.*, 2009, Luuk *et al.*, 2009). The reduced body weight has also been demonstrated in the conditional *Wfs1* mouse model at 5-6 months of age (Riggs *et al.*, 2005). The *Wfs1*-ex5-KO232 rats display reduced growth and body weight starting from 4 months of age (Plaas *et al.*, 2017). Insufficient data is available on the body parameters of WFS patients and even less is known about heterozygous *WFS1* mutation carriers (Simsek *et al.*, 2003). As mentioned, chronic overfeeding causing weight gain and obesity increase the risk of developing metabolic syndrome and other chronic diseases, for example T2D, leading to premature death (Brown *et al.*, 2009, Guh *et al.*, 2009).

HFD feeding induces besides obesity also glucose intolerance and β-cell dysfunction. Insulin resistance is often associated with obesity and leads to further complications and increased insulin need in turn promoting β-cell dysfunction and reduction of β-cell mass. Defective insulin secretion is an early indication of glucose-stimulated insulin secretion impairment (Collins *et al.*, 2010, Giacca *et al.*, 2011).

In paper IV (Ivask *et al.*, 2021) HFD caused the blood glucose level to be higher for a longer time in the glucose tolerance test (GTT), indicating an impaired glucose tolerance following HFD, however, this was present in Wfs1HZ as well as in WT mice (Fig. 17). In addition, also the plasma insulin levels or number of pancreatic islets did not differ (Fig. 18-19) (Ivask *et al.*, 2021). The low fat diet (LFD) group GTT results (Fig. 17) were in accordance with previously published studies, where Wfs1HZ mice had a GTT curve similar to WT mice (Noormets *et al.*, 2014, Punapart *et al.*, 2014, Sedman *et al.*, 2016, Terasmaa *et al.*, 2011). The same studies also reported that plasma insulin levels were not different between Wfs1HZ and WT mice (Noormets *et al.*, 2014, Terasmaa *et al.*, 2011).

Hyperglycemia has a key role in triggering the processes leading to β-cell dysfunction in case of HFD. It has been shown that acute hyperglycemia causes β-cell compensation, while chronic hyperglycemia leads to β-cell exhaustion, dysfunction and even death (Cerf, 2015). It has been proposed that non-esterified, long-chain fatty acids could potentiate insulin secretion following glucose stimulation by stimulating the glucose effect on insulin secretion instead of direct stimulation (Crespin *et al.*, 1969, Poitout, 2018). In paper IV

(Ivask *et al.*, 2021) the insulin secretion results showed large variation, indicating both HFD and genotype dependent changes in the insulin secretion function (Fig. 20). Although HFD increased the normalized insulin ratio following glucose stimulation, especially in Wfs1HZ mice (Fig. 20), similar effects were not observed for glucose tolerance, plasma insulin levels and number of pancreatic islets (Fig. 17-19). It was hypothesized that fluctuating responses could reflect the sensitivity of Wfs1HZ as a borderline genotype between Wfs1KO and WT mice (Ivask *et al.*, 2021).

HFD animals exhibit progression of ER stress, leading to protein degradation and autophagy because of increased oxidative stress and mitochondrial dysfunction (Yuzefovych *et al.*, 2013). Saturated fatty acids, that are predominant in lard, have been shown to induce ER stress more than for example polyunsaturated fatty acids, altering ER membrane composition and impairing membrane function (Fernandes-Da-Silva *et al.*, 2021, Zhao *et al.*, 2013). Lipids have been shown to modify membrane functions regulated by IRE1α and PERK, therefore inducing ER stress. In addition, the $Ca^{2+}$ permeability could be altered leading to mitochondrial dysfunction (Lee and Min, 2018). In case of WFS it has been shown that increased level of ER stress due to WFS1 deficiency is a critical molecular mechanism underlying cell death and progression of WFS symptoms (Fonseca *et al.*, 2005, Fonseca *et al.*, 2010, Kakiuchi *et al.*, 2009). Hence, in paper IV (Ivask *et al.*, 2021) the expression ER stress marker genes was determined and the expression patterns varied between genotypes, diets and tissues. It was hypothesized that because of the genetic predisposition to elevated ER stress, the expression of ER stress genes is more evident in Wfs1HZ mice and especially after HFD exposure. HFD seemed to mainly affect liver (Fig. 21) and pancreatic islets (Fig. 24) that are considered metabolically important tissues and associated with energy balance including fat and insulin metabolism. However, the expression of several ER stress genes was not changed between the different groups. For example, the diet type in paper IV did not affect the expression of *Atf6α*, *Xbp1*, *Jnk* and *Hspa5* (Fig. 21-24). As expected the expression of *Wfs1* was lower in Wfs1HZ, but the anticipated increased expression was not constant with HFD. The expression of *Wfs1* was reduced in the islets of Wfs1HZ confirming the connection between *Wfs1* and other ER stress genes (Fig. 24). For example, *Chop* and *Ire1α* expression in the pancreatic islets increased following HFD in Wfs1HZ (Wfs1HZ-H) (Fig. 24). HFD mostly affected *Chop* expression in the kidney (Fig. 22) and heart (Fig. 23) in both genotypes (Ivask *et al.*, 2021). In case of high levels of unresolved and prolonged ER stress apoptosis is induced via three pathways: the IRE1/ASK1/JNK pathway, the CASP12 kinase pathway and the CHOP pathway (Hetz, 2012). Abreu *et al.* (2020) showed that WFS1 has a role in the CHOP-TRIB3 pathway regulating apoptosis. Although CHOP is important for initiating apoptosis and cell death in case of the prolonged unfolded protein response, cooperation with ATF4 is also needed for cell death induction (Han *et al.*, 2013). In liver the expression of *Ire1α* and *Atf4* surprisingly decreased in Wfs1HZ following HFD (Fig. 22) (Ivask *et al.*, 2021). High variation in these

results illustrates how Wfs1HZ as a borderline genotype between Wfs1KO and WT mice.

A major limitation to paper IV (Ivask *et al.*, 2021) was that plasma insulin and triglyceride levels were not monitored in the HFD experiment. Following more parameters and having more animals in groups would have allowed better monitoring of the development of metabolic disturbances. In retrospect, it would have been interesting to also include Wfs1KO mice and/or high carbohydrate or high fat-high carbohydrate diet to see the different diet composition effects in case of WFS1 deficiency as this has not been studied.

In conclusion, the HFD influenced the metabolic parameters, like body weight, insulin secretion and the expression of some ER stress genes. The main differences between WT and Wfs1HZ animals following HFD were in insulin secretion as the normalized insulin ratio in Wfs1HZ following HFD was higher compared to WT animals. However, HFD fed WT and Wfs1HZ animals did not display many different effect sizes due to the higher variation caused by the heterozygous *Wfs1* mutation in Wfs1HZ animals. These results indicate that HFD causes more metabolic impairments and increased ER stress already with only one functional *Wfs1* gene copy compared to the LFD. Therefore, even a minor *Wfs1* gene deficiency may induce the progression of adverse metabolic impairments and progression of WFS associated symptoms.

# 7. CONCLUSIONS

The following conclusions are drawn based on the findings of the four studies presented in this thesis.

1. WFS1 deficiency causes transcriptomic changes in hypothalamus, hippocampus and pancreatic islets. These transcriptomic changes further contribute to the development and progression of the variety of endocrine, neurodegenerative and behavioral symptoms associated with WFS (Fig. 25).
2. WFS1 deficiency decreases the number of pancreatic islets and insulin secretion contributing to the diabetes-like phenotype. RNA-sequencing of WFS1-deficient pancreatic islets showed that *Trpm5* is downregulated and the pathways related to tissue morphology, endocrine system development and function, and molecular transport network are influenced. Functional interaction studies on WFS1 and TRPM5 in the regulation of insulin secretion could find additional pathogenesis mechanisms and possible treatment options (Fig 25.).
3. WFS1 deficiency induces metabolic disturbances as Wfs1KO mice weigh significantly less, have a shorter life span and have higher $O_2$ consumption, and $CO_2$ and heat production. RNA-sequencing of WFS1-deficient hippocampus showed that *Trpm8* is upregulated and therefore menthol, a TRPM8 agonist, enhanced the metabolic parameters more in Wfs1KO mice (Fig 25).
4. High fat diet causes metabolic impairments, like weight gain and insulin secretion changes, and ER stress gene expression alterations in heterozygous mice with only one functional *Wfs1* gene copy. However, *Wfs1* heterozygosity creates variation in the severity of the metabolic complications and ER stress gene expression patterns associated with adverse metabolic dysfunction and development of Wolfram syndrome related complications.

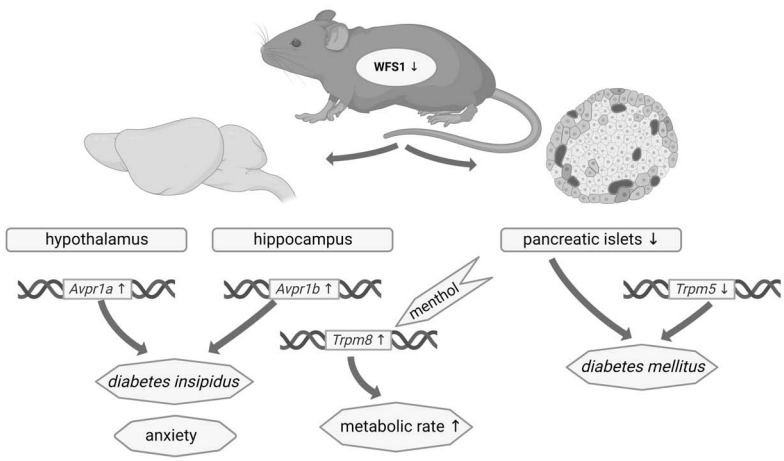


**Figure 25. Summarizing figure of conclusions 1–3.** A visual summary of conclusions 1–3 on how WFS1-deficiency may lead to development of WFS related symptoms via different genes.

# SUMMARY IN ESTONIAN

## Transkriptoomilised ja metaboolsed muutused WFS1-puudulikkusega hiiremudelis

Wolframi sündroom (WFS, OMIM 222300) on haruldane progresseeruv neurodegeneratiivne autosoom-retsessiivse pärilikkusega haigus. Haigust põhjustavad mutatsioonid volframiin-1 geenis (WFS1). Antud haigus on tuntud ka akronüümiga DIDMOAD, mis inglise keeles võtab kokku häire peamised sümptomid: juveniilne insuliinsõltuv diabeet, optiline atroofia, magediabeet ja kurtus (Barrett *et al.*, 1995, Barrett and Bundey, 1997, Inoue *et al.*, 1998, Kellner *et al.*, 1994, Strom *et al.*, 1998, Wolfram and Wagener, 1938).

WFS1 geen koosneb 8 eksonist, millest esimene on mittekodeeriv (Inoue *et al.*, 1998). Geeni 8. ekson on suurim, mistõttu on suurem osa haigusega seotud mutatsioone leitud just sellest WFS1 valgu transmembraanset ja C-terminaalset domeeni kodeerivast piirkonnast. WFS on varieeruva sümptomaatikaga haigus, sest on leitud üle paarisaja haigust põhjustava mutatsiooni ja haigusega seotud sümptomite esinemine ja raskusaste sõltub konkreetsest mutatsioonist (Cryns *et al.*, 2003, De Heredia *et al.*, 2013, Rigoli *et al.*, 2018). WFS1 valk on üheksa transmembraanse segmendiga hüdrofoobne glükoproteiin, mis paikneb peamiselt endoplasmaatilises retiikulumis (ER) (Hofmann *et al.*, 2003, Inoue *et al.*, 1998, Philbrook *et al.*, 2005, Takeda *et al.*, 2001). WFS1 on ekspresseeritud mitmetes kudedes, näiteks pankrease β-rakkudes ja ajus (Fonseca *et al.*, 2010, Hofmann *et al.*, 2003, Inoue *et al.*, 1998, Ishihara *et al.*, 2004, Strom *et al.*, 1998). WFS1 ekspressiooni häired aju limbilistes struktuurides ja hüpotaalamuses selgitavad WFS-iga seotud endokriinse regulatsiooni, kognitiivse ja emotsionaalse käitumise probleeme ning pankreases diabeediga seotud sümptomite arengut (Kato *et al.*, 2008, Luuk *et al.*, 2008, Takeda *et al.*, 2001).

WFS1 on oluline ER-i homöostaasi säilitamiseks, kuid täpne molekulaarne funktsioon vajab siiski veel uurimist. WFS1 omab mitmeid funktsioone, osaledes membraanitranspordis, valkude töötlemises, ER $Ca^{2+}$ taseme reguleerimises ja raku vastuses voltumata valkude kogunemisele (*unfolded protein response*, UPR) (Fonseca *et al.*, 2005, Fonseca *et al.*, 2010, Hatanaka *et al.*, 2011, Hofmann and Bauer, 2006, Osman *et al.*, 2003, Takeda *et al.*, 2001). Üha rohkem on teavet selle kohta, et ER homöostaasi kõrvalekalded ja krooniline ER stress on olulised mitmete haiguste arengus, näiteks vähk, maksa- ja neurodegeneratiivsed haigused ning suhkurtõbi (Gwiazda *et al.*, 2009, Hoozemans *et al.*, 2012, Ilieva *et al.*, 2007, Ozcan and Tabas, 2012, Xiang *et al.*, 2017). WFS1 on ER stressi korral tavaliselt ülesreguleeritud ning selle puudumine soodustab kroonilist ER stressi ja raku apoptoosi, mis omakorda põhjustavad WFS-ile iseloomulikke sümptomite avaldumist ja progresseerumist (Fonseca *et al.*, 2005, Fonseca *et al.*, 2009, Fonseca *et al.*, 2010, Ishihara *et al.*, 2004).

WFS molekulaarsete mehhanismide uurimiseks on loodud mitu näriliste mudelit. Käesolevas doktoritöös on kasutatud WFS hiiremudelit, mille puhul enamus *Wfs1* 8. eksonist on asendatud ja seetõttu toodetakse defektset WFS1

valku (Koks *et al.*, 2009, Luuk *et al.*, 2009). Antud töö eesmärgiks oli leida WFS1 puudulikkusest tingitud võimalikke transkriptoomilisi ja metaboolseid muutuseid, mis soodustavad WFS sümptomite arengut. RNA-sekveneerimist kasutades uuriti WFS1-puudulike hiirte hüpotaalamuse, hipokampuse ja pankrease Langerhansi saarte transkriptoomi. Lisaks uuriti ka insuliini sekretsiooni isoleeritud pankrease saarekestest, sest insuliinsõltuv diabeet on WFS üks peamine sümptom. Ainevahetushäireid, nagu 2. tüüpi diabeeti, on seostatud krooniliselt kõrgenenud ER stressiga, näiteks ülesöömise tõttu (Bhattarai *et al.*, 2020, Fernandes-Da-Silva *et al.*, 2021), ja mitmed uuringud on seostanud WFS1 mutatsioone suurenenud riskiga haigestuda 2. tüüpi diabeeti (Minton *et al.*, 2002, Sandhu *et al.*, 2007, Florez *et al.*, 2008, Franks *et al.*, 2008, Van Hoek *et al.*, 2008, Cheurfa *et al.*, 2011), seetõttu uuriti ka *Wfs1* heterosügootsete hiirte vastuvõtlikkust kõrge rasvasisaldusega dieedist põhjustatud metaboolsete häirete tekkele.

Doktoritöös leiti, et WFS1 puudulikkus põhjustab transkriptoomilisi muutusi hüpotaalamuses, hipokampuses ja pankrease Langerhansi saartes. Tulemused lubavad arvata, et need transkriptoomilised muutused soodustavad WFS-iga seotud mitmesuguste endokriinsete, neurodegeneratiivsete ja käitumuslike sümptomite teket ja progresseerumist.

WFS1 puudulikkusse korral väheneb pankrease Langerhansi saarte arv ja häirub insuliini sekretsioon. WFS1-puudulike pankrease saarekeste RNA-sekveneerimine näitas, et funktsionaalse WFS1 puudumisel on vähenenud ka geeni *Trpm5* ekspressioon. Peamiselt on mõjutatud kudede morfoloogia, endokriinsüsteemi arengu ja funktsiooni ning molekulaarse transpordivõrguga seotud signaalrajad. WFS1 ja TRPM5 omavahelist seost ei ole uuritud, kuid nende funktsionaalsete koostoimete uurimine insuliini sekretsiooni reguleerimisel aitaks paremini selgitada WFS ja diabeedi patogeneesi mehhanisme ning leida võimalikke uusi ravivõimalusi.

WFS1 puudulikkus põhjustab ka metaboolseid häireid, sest võrreldes oma normaalsete pesakonnakaaslastega kaaluvad *Wfs1* mutantsed hiired (Wfs1KO) oluliselt vähem, neil on lühem eluiga, suurem hapnikutarbimine ning süsihappegaasi ja soojuse tootmine. WFS1-puuduliku hipokampuse RNA-sekveneerimise tulemustest selgus, et Wfs1KO hiirte hipokampuses on rohkem ekspresseerunud *Trpm8* geen, mistõttu TRPM8 agonisti, mentooli, manustamine mõjutas Wfs1KO hiirte metaboolseid parameetreid rohkem.

Kõrge rasvasisaldusega dieet põhjustab ühe funktsionaalse *Wfs1* geeni koopiaga hiirtel metaboolseid häireid, nagu kaalutõus, insuliini sekretsiooni muutused ning ER stressiga seotud geenide ekspressiooni muutused. *Wfs1* heterosügootsus põhjustab aga variatsiooni nii metaboolsete tüsistuste kui ka ER stressi vahendavate geenide ekspressiooni mustrites, mis on seotud ebasoodsate metaboolsete häirete ja Wolframi sündroomi sümptomite kujunemisega. Seetõttu oleks huvitav uurida kõrge rasvasisaldusega dieedi mõju Wfs1KO hiirtel.

Kokkuvõtvalt võib järeldada, et antud doktoritöö tulemused täiendavad Wolframi sündroomi ja WFS1 geeni kohta olemasolevat infot ning annavad ainest uuteks uuringuteks.

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# ACKNOWLEDGMENTS

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# ORIGINAL PUBLICATIONS

# CURRICULUM VITAE

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**EDUCATION**

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| 2011–present | **Doctoral studies (medical science)**<br>University of Tartu, Faculty of Medicine, Department of Pathophysiology, Estonia |
| 2009–2011 | **MSc (biomedicine)**<br>University of Tartu, Faculty of Medicine, Department of Physiology, Estonia |
| 2006–2009 | **BSc (genetic engineering)**<br>University of Tartu, Faculty of Science and Technology, Estonia |

**WORK EXPERIENCE**

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| April 2014 – present | **Specialist**<br>University of Tartu, Institute of Biomedicine and Translational Medicine, Department of Pathophysiology, Estonia |
| February 2011 – present | **Senior specialist**<br>Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Chair of Animal Breeding and Biotechnology, Estonia |
| February 2017 – December 2021 | **Researcher**<br>Competence Centre on Health Technologies, Estonia |
| January 2010 – August 2015 | **Laboratory technician**<br>Competence Centre on Health Technologies, Estonia |

**ADDITIONAL COURSES**

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|---|---|
| November 2018 | **Biopsy Techniques**<br>CooperSurgical Fertility and Genomics Solution, Denmark |
| January – February 2015 | **The use of animals in research: course for persons carrying out procedures**<br>University of Helsinki, Finland |
| January 2009 | **Laboratory animal science: C-category competence course**<br>University of Tartu, Estonia |

**PUBLICATIONS**


1. Waszczykowska A., Zmyslowska A., Bartosiewicz K., Studzian M., Pulaski L., Braun M., **Ivask M**., Kõks S., Jurowski P., Mlynarski W. (2021). Reduced Corneal Sensitivity with Neuronal Degeneration is a Novel Clinical Feature in Wolfram Syndrome. *American Journal of Ophthalmology*. 236: 63–68.
2. **Ivask M**., Volke V., Raasmaja A., Kõks S. (2021). High-fat diet associated sensitization to metabolic stress in Wfs1 heterozygous mice. *Mol Genet Metab*. 21: 00753–8.
3. Waszczykowska A., Zmyslowska A., Braun M., **Ivask M**., Kõks S., Jurowski P., Mlynarski W. (2020). Multiple Retinal Anomalies in Wfs1-Deficient Mice. *Diagnostics*. 10(9): 607.
4. Waszczykowska A., Zmyslowska A., Braun M., Zielonka E., **Ivask M**., Kõks S., Jurowski P., Mlynarski W. (2020). Corneal Abnormalities Are Novel Clinical Feature in Wolfram Syndrome. *American Journal of Ophthalmology*. 217: 140–151.
5. Eimre M., Paju K., Peet N., Kadaja L., Tarrend M., Kasvandik S., Seppet J., **Ivask M**., Orlova E., Kõks S. (2018). Increased Mitochondrial Protein Levels and Bioenergetics in the Musculus Rectus Femoris of Wfs1-Deficient Mice. *Oxid Med Cell Longev*. 2018: 3175313.
6. Eimre M., Kasvandik S., **Ivask M**., Kõks S. (2018). Proteomic dataset of wolframin-deficient mouse heart and skeletal muscles. *Data Brief*. 21: 616–619.
7. Li D., Secher J., Hyttel P., **Ivask M**., Kolko M., Hall V.J., Freude K.K. (2018). Generation of transgene-free porcine intermediate type induced pluripotent stem cells. *Cell Cycle*. 17(23): 2547–2563.
8. **Ivask M**., Pajusalu S., Reimann E., Kõks S. (2018). Hippocampus and Hypothalamus RNA-sequencing of WFS1-deficient Mice. *Neuroscience*. 374: 91–103.
9. **Ivask M**., Hugill A., Kõks S. (2016). RNA-sequencing of WFS1-deficient pancreatic islets. *Physiol Rep*. 4(7): e12750.
10. Ehrlich M., **Ivask M**., Raasmaja A., Kõks S. (2016). Analysis of metabolic effects of menthol on WFS1-deficient mice. *Physiol Rep*. 4(1): e12660.
11. Noormets K., Kõks S., **Ivask M**., Aunapuu M., Arend A., Vasar E., Tillmann V. (2014). Energy metabolism and thyroid function of mice with deleted wolfram in (Wfs1) gene. *Exp Clin Endocrinol Diabetes*. 122(5): 281–6.
12. Kõks S., Overall R.W., **Ivask M**., Soomets U., Guha M., Vasar E., Fernandes C., Schalkwyk L.C. (2013). Silencing of the WFS1 gene in HEK cells induces pathways related to neurodegeneration and mitochondrial damage. *Physiological Genomics*. 45(5): 182–190.

**INDUSTRIAL PROPERTY**

Invention: A method of producing biotechnological drugs using transgenic bovine; Owners: Competence Centre on Health Technologies; Authors: Sulev Kõks, Mario Plaas, Pille Pärn, **Marilin Ivask**, Monika Nõmm, Jevgeni Kurõkin, Riho Meier, Ülle Jaakma, Ene Reimann, Rutt Lilleoja, Aili Tagoma; Priority number: 14193488.5; Priority date: 17.11.2014.

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**PUBLIKATSIOONID**


1. Waszczykowska A., Zmyslowska A., Bartosiewicz K., Studzian M., Pulaski L., Braun M., **Ivask M**., Kõks S., Jurowski P., Mlynarski W. (2021). Reduced Corneal Sensitivity with Neuronal Degeneration is a Novel Clinical Feature in Wolfram Syndrome. *American Journal of Ophthalmology*. 236: 63–68.
2. **Ivask M**., Volke V., Raasmaja A., Kõks S. (2021). High-fat diet associated sensitization to metabolic stress in Wfs1 heterozygous mice. *Mol Genet Metab*. 21: 00753–8.
3. Waszczykowska A., Zmyslowska A., Braun M., **Ivask M**., Kõks S., Jurowski P., Mlynarski W. (2020). Multiple Retinal Anomalies in Wfs1-Deficient Mice. *Diagnostics*. 10(9): 607.
4. Waszczykowska A., Zmyslowska A., Braun M., Zielonka E., **Ivask M**., Kõks S., Jurowski P., Mlynarski W. (2020). Corneal Abnormalities Are Novel Clinical Feature in Wolfram Syndrome. *American Journal of Ophthalmology*. 217: 140–151.
5. Eimre M., Paju K., Peet N., Kadaja L., Tarrend M., Kasvandik S., Seppet J., **Ivask M**., Orlova E., Kõks S. (2018). Increased Mitochondrial Protein Levels and Bioenergetics in the Musculus Rectus Femoris of Wfs1-Deficient Mice. *Oxid Med Cell Longev*. 2018: 3175313.
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7. Li D., Secher J., Hyttel P., **Ivask M**., Kolko M., Hall V.J., Freude K.K. (2018). Generation of transgene-free porcine intermediate type induced pluripotent stem cells. *Cell Cycle*. 17(23): 2547–2563.
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10. Ehrlich M., **Ivask M**., Raasmaja A., Kõks S. (2016). Analysis of metabolic effects of menthol on WFS1-deficient mice. *Physiol Rep*. 4(1): e12660.
11. Noormets K., Kõks S., **Ivask M**., Aunapuu M., Arend A., Vasar E., Tillmann V. (2014). Energy metabolism and thyroid function of mice with deleted wolfram in (Wfs1) gene. *Exp Clin Endocrinol Diabetes*. 122(5): 281–6.
12. Kõks S., Overall R.W., **Ivask M**., Soomets U., Guha M., Vasar E., Fernandes C., Schalkwyk L.C. (2013). Silencing of the WFS1 gene in HEK cells induces pathways related to neurodegeneration and mitochondrial damage. *Physiological Genomics*. 45(5): 182–190.

**TÖÖSTUSOMAND**

Patentne leiutis: A method of producing biotechnological drugs using transgenic bovine; Omanikud: Tervisetehnoloogiate Arenduskeskus AS (endine Reproduktiivmeditsiini TAK AS); Autorid: Sulev Kõks, Mario Plaas, Pille Pärn, **Marilin Ivask**, Monika Nõmm, Jevgeni Kurõkin, Riho Meier, Ülle Jaakma, Ene Reimann, Rutt Lilleoja, Aili Tagoma; Prioriteedi number: 14193488.5; Prioriteedi kuupäev: 17.11.2014.

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