Proteomic and Phosphoproteomic Analysis of the effect of Exendin-4 Treatment on the Rat Pancreatic Beta Cell Line Ins-1

Department of Diabetes and Endocrinology

Faculty of Medicine

Imperial College in London

MPhil Thesis

Rasha Al Mismar

Supervised by

Dr. Kevin Murphy

Dr. Johannes Graumann

Dr. Shahrad Taheri

Declaration of Originality

I declare that this thesis is my own work and that, to the best of my knowledge, the results presented here have not been previously published or produced by other parties.

Copyright Declaration

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Abstract

Diabetes is a major worldwide health problem, and the incidence is increasing. Although the etiology of types 1 and 2 diabetes is different, the developmental complications of both types involve destruction of pancreatic beta cells. Enhancing or inducing pancreatic beta cell proliferation is thus a focus of much research. Exendin-4, a GLP-1 analogue, was introduced into the clinic in 2005 as an inducer of insulin secretion, but also has proliferative effects on pancreatic beta cells *in vitro*. These effects have been characterized in targeted studies, identifying specific proteins in particular signaling pathways. In our study, we aimed to carry out global proteomic and phosphoproteomic relative quantitation screening to identify the proteins and their phosphorylation sites regulated by exendin-4 treatment in rat ins-1 cells, compared to their untreated counterparts. We were able to quantify 3766 proteins among which 667 proteins were significantly regulated. In addition, we quantified 2170 phosphosites, 400 of which were significantly regulated. The significantly regulated proteins and phosphosites after exendin-4 treatment pointed to a protective role for this peptide in maintaining cell survival and inhibiting apoptosis due to the stress induced by the serum starvation, which is in agreement of previous reports. This thesis thus provides the first global proteomic analysis of the effects of exendin-4 on the rat pancreatic beta cells. Further studies employing different culture conditions and knockdown or overexpression of candidate proteins are now required to give deeper insight into the pathways involved.

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I. Diabetes

A. Definition

Diabetes mellitus is a chronic disease state characterized by the accumulation of glucose in the blood due to the inability of the body cells to produce and/or use insulin. It is a heterogeneous disease associated with multiple metabolic disorders. The sustained high level of blood glucose due to the insufficient insulin release or the inability of body cells to use it causes damage to several body parts including the heart, kidney, blood vessels and eyes leading to hypertension and consequently shorter life expectancy (1).

B. Pathophysiology and Complications

Understanding the pathophysiology of diabetes relies on a fundamental understanding of glucose metabolism and insulin action. In normal healthy individuals, carbohydrates from food are broken down into glucose which is absorbed into the blood stream to be utilized by body cells such as the muscle cells. High blood glucose is sensed by the pancreas, where the islet beta cells secrete insulin in response to high glucose levels. This secreted insulin aids the glucose entry into the cells. As the body cells take up the glucose, its level decreases in the blood causing pancreatic cells to stop producing insulin to maintain glucose homeostasis.

Perturbations to the above process, such as diabetes mellitus, will affect blood glucose dynamics. If insulin is not secreted by pancreatic beta cells in response to high glucose levels or insulin is normally secreted but not able to exert its effects, cells will not be able to take up the glucose which will accumulate in the blood leading to hyperglycemia and other complications. Inversely, if excess insulin is produced, more glucose enters the cells leading to low levels in the blood stream and hypoglycemia.

The pancreas plays a major role in maintaining blood glucose homeostasis because it contains Islets of Langerhans, which are scattered across the pancreas and are made of several types of cells that work in coordination to maintain normal glucose levels: alpha, beta, gamma, delta

and epsilon cells. Beta islets are the cells responsible for insulin secretion in response to high blood glucose levels. In cases of hypoglycemia, alpha islets secrete the hormone glucagon which causes the breakdown of glycogen in the liver into glucose. Glycogen is a polysaccharide made of glucose molecules and stored in the liver and muscles as a form of energy.

Diabetes mellitus is a chronic disease which manifests into several macrovascular and microvascular complications (2). The latter are diabetic nephropathy, neuropathy, and retinopathy while the macrovascular complications include coronary artery disease, peripheral arterial disease, and stroke (2).

C. Types of Diabetes

i. Type 1

Type 1 diabetes mellitus (T1DM), previously known as insulin-dependent or juvenile diabetes, accounts for 5-10% of all cases of diabetes mellitus (3). It is characterized as an autoimmune disease affecting pancreatic beta cells and impairing their ability to produce insulin. Its onset is typically early in childhood, and the precise causes are unknown. Type 1 patients need insulin injection to survive and there are no known preventive measures (1). The disease manifests after infiltration of the pancreas with antibodies directed against the several antigens on the beta islets of Langerhans (4). The autoantibodies detected in the pancreas are directed against molecules including insulin, glutamic acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2) and zinc transporter 8 (ZNT8) (5). The continuous recruitment of T lymphocytes and macrophages leads to the production of cytokines and chemokines which eventually leads to apoptosis of the beta cells. The mechanism by which the first autoantibody is produced and directed against the beta cells is unknown (5). The disease may remain asymptomatic for years during which autoimmune destruction of the beta cells leads to their inability to produce insulin and to disease onset, causing 75% beta cell destruction at diagnosis (4). Although type 1 diabetes etiology is thought to be solely genetic, an identical twin has approximately 50% chance of developing type 1 diabetes if their sibling is affected, and consequently, environmental, nutritional and viral factors have been proposed to play a role in disease development (6).

ii. Type 2

Type 2 diabetes mellitus (T2DM) is the most common type of diabetes mellitus and was previously known as insulin-independent diabetes. It is characterized by insufficient insulin to maintain normal glucose homeostasis in the face of insulin resistance. As a result, insulin production becomes insufficient over time, as resistance increases and insulin-producing beta cells succumb to chronic cellular stress caused by insulin overproduction stemming from the attempt to counteract resistance, with approximately 50% of the beta cell capacity lost by the time of diagnosis (7). The lack of sensitivity to insulin in the periphery blocks the entry of glucose into the cells leading to its accumulation in the blood stream. T2DM was still regarded only a few years ago as the disease of insulin resistance. However, studies have suggested that impaired insulin secretion in type 2 diabetics, caused by the inability of the beta cell to compensate for insulin resistance, is the major driver of T2DM (3). The disease is mainly controlled by medication and limiting sugar consumption in the diet. T2DM is a multifactorial disease where obesity, life style, age and genetic factors play a major role in its development (7). One of the most deleterious consequences of T2DM is cardiovascular disease (CVD) (8).

iii. Gestational Diabetes

Gestational diabetes mellitus (GDM) is the type of diabetes that appears during pregnancy. However, it has been proposed that GDM could be viewed as an early stage of T2DM arising during pregnancy (9). Obesity and increased pregnancy age of the mother predispose the pregnant women to this type of diabetes. The main etiology of the disease is the change in the metabolic state of the pregnant human body, especially at the late stages of gestation. This is thought to be due to hormones secreted by the placenta causing peripheral insulin resistance. If insulin secretion does not rise to a level that overcomes the peripheral resistance, the risk of developing gestational diabetes becomes very high (9). These effects are usually alleviated after delivery but the mother and the child remain at high risk of developing obesity and T2DM later in life (9).

iv. Latent Autoimmune Diabetes in Adults (LADA)

LADA is the type of diabetes combining characteristics from both T1DM and T2DM where 5-14% of the patients have pancreatic autoantibodies and require insulin injections while the rest exhibit T2DM phenotype except for reduced incidence of obesity and metabolic syndrome (10).

v. Neonatal Diabetes

Neonatal diabetes (ND) arises within the first 6 months of life and is distinguished from T1DM by the lack of pancreatic autoantibodies and by being a monogenic disease (3). Recently, mutations in several genes have been linked to ND, including the genes encoding glucokinase and the K_{ATP} channel (11). The incidence of the disease is low and it can be either transient or chronic (11). Mutations in the glucokinase and hepatic nuclear factor 1 alpha (HNF1A) genes account for about 70% of cases (3). The disease usually remains controlled by diet.

D. Statistics

According to the International Diabetes Federation (IDF): "Diabetes is one of the largest global health emergencies of the 21st century". Each year more and more people live with this condition, which can result in life-changing complications". According to the IDF atlas, 415 million adults are estimated to currently have diabetes, more than 90% of whom have type 2 diabetes, and 318 million are at high risk of developing the disease. In 2015, it was calculated that 1 in 11 adults have diabetes, with higher prevalence in men than in women. This ratio is expected to be 1 in 10 adults by 2040 (12).

In the United States, data collected by the Centers for Disease Control and Prevention indicate that nearly 26 million people have diabetes, with the vast majority having T2DM (8). As mentioned earlier, CVD is among the most serious consequences of T2DM and is the leading cause of mortality in these patients.

Only 10-15% of all diabetic patients have T1DM; it is the most common form of diabetes in children below 15 years, and, according to the IDF, more than 500,000 children are currently

living with this disease (12). Table 1, taken from IDF, lists the incidents of diabetes around the world and the anticipated increase by 2035.

Table 1: Diabetes incidence around the world in 2013 and the anticipated rise in 2035

IDF REGION	2013 MILLIONS	2035 MILLIONS	INCREASE %
Africa	19.8	41.4	109%
Middle East and North Africa	34.6	67.9	96%
South-East Asia	72.1	123	71%
South and Central America	24.1	38.5	60%
Western Pacific	138.2	201.8	46%
North America and Caribbean	36.7	50.4	37%
Europe	56.3	68.9	22%
World	**381.8**	**591.9**	**55%**

E. Treatments and limitations

Several drugs acting as enhancers of insulin secretion and reducers of insulin resistance have been introduced into the clinic and used as treatments for diabetes, either as monotherapy or a combination of pharmacological interventions. Insulin, metformin, sulphonylureas, thiazolidiones (TZD), sodium-glucose co-transporter 2 (SGLT2) inhibitors, bile acid sequestrants, dipeptidyl peptidase-4 (DPP-4) inhibitors, α-glucosidase inhibitors and glucagon-like peptide-1 receptor (GLP1-R) agonists are among the treatments used (13,14).

Metformin decreases hepatic glucose production and induces weight loss, while TZDs cause weight gain but improve insulin sensitivity. Sulfonylureas enhance insulin secretion while SGLT2 inhibitors alter renal glucose reabsorption, resulting in more glucose excretion in the urine (14). Bile acid sequesterants are known to decrease fasting blood glucose levels and to induce secretion of incretins like GLP-1 (15). GLP-1 receptor agonists induce insulin secretion and improve β-cell function. DPP-4 inhibitors inhibit enzymes that degrade GLP-1, consequently increasing its circulating concentrations and inducing glucose-dependent insulin secretion and inhibiting glucagon release (14).

The above listed pharmacological treatments have side effects characterized mainly by induction of hypoglycemia, especially if administered in combination, and changes in body

weight. GLP-1 receptor agonists seem to stabilize blood glucose levels but are associated with loss of body weight, in addition to nausea and vomiting, though this can be controlled by escalating dosage gradually (7).

While pharmacological treatment, bariatric surgery and diet could be effective treatments for T2DM, T1DM requires another level of intervention to correct for beta cell destruction. Pancreatic transplantation came into practice in the last fifty years, with the first successful whole organ transplantation done in 1966 in the US (16). The number of pancreatic transplants increased to 9000 cases worldwide by the year 1996 (16). Despite the success rate of this procedure and the correction of blood glucose levels, there are major limitations to it, including high cost, need for immunosuppressive drugs and limited number of donors. Islet transplantation, besides being a less invasive procedure, results in a lower rate of long term insulin independent euglycemia compared to whole organ transplant, with the costs being comparable between the two procedures (16).

Stem cell research has recently been aiming at the replacement of non-functional beta cells in T1DM and T2DM, and there have been several successful attempts in the lab to generate insulin secreting pancreatic cells from embryonic stem cells and induced pluripotent stem cells (iPSC) (17). However, this approach holds risks and limitations, especially in terms of the ethical use of human stem cells and the potential of developing teratomas. The use of iPSCs in generating pancreatic beta cells is thought to be much safer, but protocols for producing islet-like cells that stabilize blood glucose levels are yet to be established (17). A group in Harvard Stem Cell Institute was able to differentiate human pluripotent stem cells into insulin-secreting pancreatic beta cells that restored blood glucose levels when transplanted into mice. However, the level of insulin secreted was much lower than that of isolated human islets (18).

Another approach to the treatment of types 1 and 2 diabetes is the induction of beta cell proliferation which will be discussed in the following section.

Induction of Beta Cell Proliferation

There is controversy as to whether pancreatic beta cells divide or differentiate from existing precursor cells lining the pancreatic duct. The adult human pancreas contains around 1-2 grams of beta cells (19). Pancreatic beta cells are known to be mitotically active at neonatal stages of life and to remain in that state for up to one year of age (19). Evidence has pointed to the possibility of beta cell formation in T1DM due to the presence of detectable C-peptide levels in 16% of patients with this disease. Others hypothesize that these cells are the result of differentiation from existing pancreatic stem cells (20). Meier *et al.* (2005) conducted several immunostaining experiments on pancreatic tissue isolated from 42 subjects with T1DM at autopsy (21). Beta cells were identified in 88% percent of the diabetic tissue regardless of the lifespan of the disease. They did not see any evidence for replication using the ki67 proliferation marker. They concluded that there is new beta cell formation as a response to the apoptosis induced by the disease but they did not investigate whether the basis of this formation is replication or differentiation (21). *In vitro*, it has been recently shown that several factors drive human, rat and mice beta cell proliferation (22). We shall discuss these factors in details later in this section. Despite the effective and serious attempts in inducing proliferation in mouse, rat and human beta cells, limitations to such efforts still exist. Many of the agents discussed below are not beta cell specific and the risk of inducing proliferation in other cell types is high. In addition, the lack of human studies testing the mitotic effects of potential agents is a barrier that remains to be overcome.

The resistance of adult beta cells to proliferation is due to cell cycle kinetics which are regulated by the expression and localization of cyclin-related proteins. In adult human beta cells, expression of p16, p26, p27 and cyclin D3, which suppress cell cycle progression, are up-regulated. In addition, the expression of duodenal homeobox-1 (PDX-1), a basic transcription factor in beta cell proliferation, is down-regulated (23). A study conducted in 2013 by Fiaschi-Taesch and colleagues reported the confinement of cyclins and their dependent kinases in human beta cells to the cytoplasm, an observation that could be a basis for future investigation

of the possibility of driving beta cell proliferation by inducing the translocation of these cyclins to the nucleus (24).

Progress in the generation of beta cells and the stimulation of their proliferation has been achieved mainly through characterizing new growth factors and signaling pathways. Recent high-throughput screening studies have shown a promising role for a group of chemical compounds in driving beta cell proliferation.

Wang P and colleagues described a high-throughput screen of chemical inducers that were able to promote the proliferation of growth-arrested mouse β cells (25). The chemical inducers exerted their effects through several signaling pathways including Wnt and L-type calcium channel (LTCC) signaling. The latter pathway activated Ras signaling and increased expression of cell cycle regulators (25). They were able to subsequently show that one of the proliferative small molecules, diarylamide WS6, induced proliferation of rodent and human islets *in vitro* and mouse β cells *in vivo* (26). Building upon this work, the same group described a new series of compounds, the aminopyrazines, that act as inhibitors of the nuclear factor of activated T-cell (NFAT) kinases, dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) and glycogen synthase kinase-3 beta (GSK3B) which eventually leads to adult primary human β-cell proliferation *in vitro* and *in vivo*, and improves glycemic control of diabetic mouse models (27). Melton and his group performed another screen and were able to identify a compound which acts as an adenosine kinase inhibitor and was able to specifically increase beta cell division in freshly isolated rat, mouse and porcine islets (28).

Using another approach utilizing the human hepatocyte cell line, HepG2, stably expressing luciferase reporter induced under the human MYC promoter, Wang and colleagues isolated candidate molecules exhibiting mitogenic effects on beta cells using chemical libraries. Among these compounds is harmine, which was able to induce human and rat β-cell proliferation both *in vitro* and *in vivo* by prompting cell cycle entry in a mode similar to that reported previously and discussed above (19,26).

In addition to the pharmacological agents, endogenous molecules and proteins have long been investigated for their effect on beta cell proliferation. Hepatocyte growth factor (HGF), also known as Nodal, is a paracrine hormone secreted by mesenchymal cells and acts on epithelial

cells (29). It was shown that overexpression of HGF in transgenic mice stimulated islet proliferation and enhanced glucose stimulated insulin secretion (GSIS) from these islets (30). The same group later identified a role for HGF/c-met signaling in inducing beta cell growth, especially following beta cell ablation (31). Another study done in 2002 reported that the increase in proliferation in pancreatic rat ins-1 cells by HGF was through activation of the JAK-2/STAT-5 pathway and subsequent activation of phosphatidylinositol-3'-kinase (PI3k). The effect of HGF was augmented by the addition of growth hormone (GH) and insulin-like growth factor 1 (IGF-1) (32). IGF-1 has been shown to induce the proliferation of the rat ins-1 cell in a glucose dependent manner (33). The same group reported that together with glucose, IGF-1, but not transforming growth factor (TGF)-α- or epidermal growth factor (EGF), was able to induce a prolonged activation of the Erk1/2 or PI3K and p70S6K through the insulin receptor substrate 2 (Irs2), thus leading to proliferation of rat ins-1 cells (34). The pregnancy hormone prolactin, which belongs to the lactogenic family of hormones, has been shown to regulate beta cell growth; prolactin receptor deficient mice have decreased beta cell mass and insulin production (35). In addition, osteoprotegerin (OPG), a target of prolactin required for prolactin mediated beta cell induction in rodents, stimulated the growth of human islets *in vitro* (36).

Serpin B1 belongs to a superfamily of proteins known as serine protease inhibitors (Serpins). Until recently, Serpin B1 was reported to inhibit the function of leukocyte elastase, cathepsin G and proteinase-3, thus protecting cells from damage at inflammatory sites (37,38). A recent study by El Ouaamari *et al.* (39) also provided evidence that Serpin B1 is involved in glucose homeostasis. They identified Serpin B1 as the factor driving the humoral cross talk between the liver and the pancreas. The study showed that Serpin B1 promotes human, mice and zebrafish pancreatic cell growth through the activation of proteins in the growth factor signaling pathway, without having a direct effect on insulin secretion. Phosphoproteomic analysis revealed increased phosphorylation of proteins involved in cell survival, particularly c-AMP dependent proteins (39). The levels of Serpin B1 were elevated in the serum of mice with insulin resistance (39), which may reflect a response to promote beta cell survival.

Irisin is a newly discovered cytokine released from skeletal muscle in humans and mice after exercise (40). Recently, Liu and colleagues conducted a study on the effect of Irisin on rat ins-1

cells. Irisin increased the proliferation of these cells via the ERK and p38 MAPK signaling pathways and protected them from high-glucose-induced apoptosis. When injected into type 2 diabetic rats, Irisin reduced their body weight and enhanced their glucose tolerance (41).

GLP-1 is an incretin hormone secreted by intestinal L cells in response to food intake. The hormone has both insulinotropic and proliferative effects on pancreatic cells in mice, rat and humans (42). It has been extensively studied and we will discuss, in detail, its actions and role in beta cell growth in the following section, along with its analogues. GLP-1 is known for its ability to induce insulin secretion and its analogues are being prescribed as medications in the clinic. In addition, the effect of GLP-1 and its analogues on the proliferation of beta cells have shown promising results that warrant further studying to identify its mechanism of action. Table 2 summarizes the agents discussed above, in addition to GLP-1, and their mode of action.

Table 2: Beta Cell Proliferating Agents

Agent	Cell model	Mode of action
Pharmacologic		
Diarylamide WS6	mouse β cells *in vivo* rat and human islets *in vitro*	Activation of Erb3 binding protein-1 and IκB kinase IKKε (26)
Aminopyrazines	human β-cell *in vitro* and *in vivo*	Inhibition DYRK1A and GSK3B (27)
Adenosine kinase inhibitor	rat, mouse and porcine islets *in vitro* mouse β cells *in vivo* rat ins-1 cell line	mTOR signaling (28)
Harmine	human and rat β-cells both *in vitro* and *in vivo*	Inhibition of DYRK1A (19)
Hormonal and enzymatic		
Prolactin	mouse islets *in vitro*	receptor knockout (35)
Osteoprotegerin	human islets *in vitro* rat islets *in vitro* and *in vivo*	Inhibition of the receptor activator of NF-kB ligand pathway (36)
GLP-1/analogues	rat ins-1 cell line mouse and rat islets *in vitro* human islets *in vitro*	cAMP/PKA mediated cyclin D expression (43) EGFR transactivation by c-Src (44) FoxO1 acetylation by inhibiting SirT1 (45) PKCζ activation (46) Activation of transcription factor Pax4 (47) AMPK/mTOR/p70S6K/4EBP (48) Up-regulation of bcl-2 and downregulation of caspase 3
Serpin B1	human islets in vivo mice and zebrafish islets *in vitro* and *in vivo*	Altered phosphorylation of MAPK, PRKAR2B, and GSK3 (39)
Irisin	rat ins-1 cell line	ERK and p38 MAPK signaling pathways (41)
Growth factors		
HGF	mouse islets *in vitro* and *in vivo*	Receptor knockout (31)
IGF-1	rat ins-1 cell line	Activating PI3k and ERK1/2 through Irs2 (34)

II. GLP-1 and its Analogues

A. Insulinotropic Effects

GLP-1 is an incretin hormone secreted by intestinal L cells in response to food intake. It has both insulinotropic and proliferative effects on pancreatic cells in the mouse, rat and human (49). GLP-1 controls blood glucose levels through independent gastrointestinal and pancreatic routes (50). After a meal, intestinal cells secrete GLP-1, inhibiting gastric emptying, resulting in delayed glucose absorption in the intestine and consequently a slower rate of glucose uptake into the blood. In addition, GLP-1 induces glucose-dependent insulin secretion from the pancreas while inhibiting glucagon secretion (50).

To understand how GLP-1 induces insulin secretion, it is crucial to understand the natural mechanism of insulin secretion induced by glucose in pancreatic beta cells. When glucose binds to its transporter GLUT-2 on pancreatic beta cell membrane, it activates glycolysis which produces ATP that blocks potassium channels resulting in membrane depolarization and the opening of the voltage-gated calcium channels which allows calcium influx into the cell. This influx stimulates the mobilization of the insulin granules and fusion with the plasma membrane leading to exocytosis. Insulin granules categorize into two pools: the readily releasable pool (RRP) and the reserve pool (RP), where the former constitutes around 1-5 % of the pool population and latter occupies the rest (51). The naming stems mainly from the localization of these granules: the RRP resides close to the plasma membrane and releases its content immediately after calcium influx, whereas the RP is further away and needs to be primed and mobilized in order to undergo exocytosis. Figure 1 summarizes the insulin secretory pathway activated upon glucose stimulation.

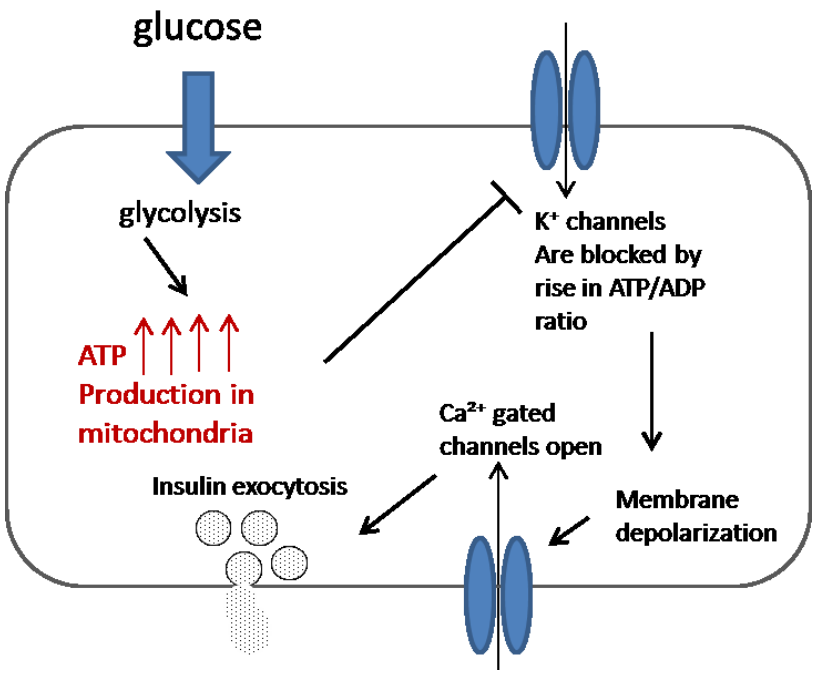

Figure 1: Insulin release from pancreatic beta cell after glucose stimulation

GLP-1 is believed to exert its glucose-dependent insulinotropic effects on pancreatic cells through binding to the GLP-1 receptor (GLP-1R) expressed in these cells. GLP-1R is a seven transmembrane G-protein coupled receptor (52). Upon binding of the ligand to this receptor, the latter interacts with a trimeric G-protein complex, resulting in adenylate cyclase activation and the production of cyclic adenosine monophosphate (cAMP), which in turn acts as a secondary messenger (53). In agreement with this model, a study on rat islets and cell lines showed that insulin secretion in response to glucose stimulation decreased by 40% in islets overexpressing cAMP-degrading phosphodiesterase (PDE) (54). A later study by Knoch *et al*., showed that the phosphorylation of PTB1 (polypyrimidine tract binding protein 1) by cAMP is key to the posttranscriptional regulation of insulin secretory granules (55). Activation of the cAMP induces insulin release through protein kinase A (PKA)-dependent and independent pathways (42). The former pathway induces several molecular mechanisms including increase in Ca^{+} influx through gated channels leading to induction of exocytosis (42). The latter pathway is mediated by the cAMP-regulated guanine nucleotide exchange factor (Epac2) pathway. Epac2 is a cAMP binding protein that induces insulin secretion by antagonizing potassium channels and mobilizing calcium from intracellular stores, which results in induction of exocytosis

(42,56). Figure 2 summarizes the pathways driven by GLP-1 for insulin production in pancreatic beta cells.

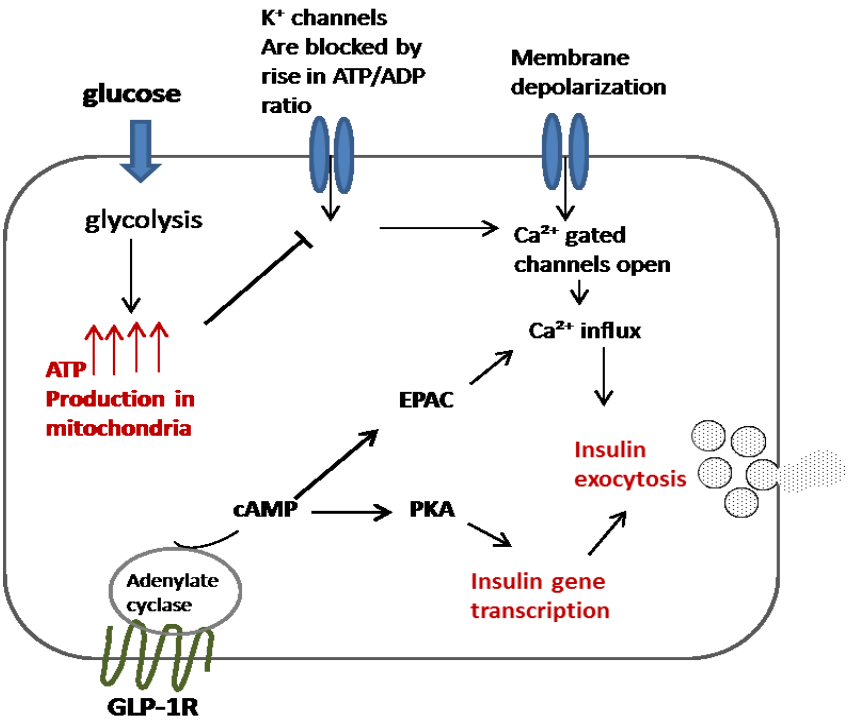

Figure 2: Insulin release from pancreatic beta cell after GLP-1R stimulation

GLP-1 is a short acting peptide with a half-life of around 1-2 minutes mainly due to degradation by the dipeptidyl peptidase 4 (DDP-4) (49), hence, it cannot be used as an antidiabetic treatment. Five of the synthesized GLP-1 analogues that are resistant to DDP-4 are nowadays approved for type 2 diabetes treatment by US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (49). These analogues are exenatide (Byetta), liraglutide (Victoza), lixisenatide (Lyxumia), albiglutide (Tanzeum or Eperzam) and dulaglutide (Trulicity). Exenatide is a synthetic analogue of exendin-4 which is extracted from Gila monster Heloderma suspectum, and shares 53% sequence homology with GLP-1 (57). We will focus in the following sections on exenatide since it was introduced into the clinic in 2005 (4) and has been extensively studied for its insulinotropic and proliferative capacities in beta cells. The terms exendin-4 and exenatide will be used interchangeably in this section.

B. Proliferative effects

The proliferative effect of GLP-1 and its agonists on pancreatic beta cells has long been investigated. This proliferative effect is driven by induction of cell replication and maintenance of cell survival (58). Buteau *et al*. have shown that GLP-1 enhances DNA synthesis in rat pancreatic beta cells through activating phosphatidylinositol 3-kinase (PI3K), an enzyme known to be involved in a cascade of phosphorylation events leading to activation of transcription factors including pancreatic/duodenal homeobox 1 (PDX-1) (59,60). They hypothesized that the GLP-1-induced phosphoinositide 3-kinase (PI3k) activity occurs through epidermal growth factor receptor (EGFR) transactivation via proto-oncogene tyrosine-protein kinase Src (c-Src) (44). The same group later reported a role for protein kinase zeta (PKCζ) activation in GLP-1-mediated beta cell proliferation in rat cells (46). Transcript levels of PDX-1 were elevated after GLP-1 treatment and its DNA binding activity was enhanced, independent of the paracrine pathway induced by GLP-1 (59). GLP-1 induced the activation of p38 mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase B (PKB) in rat cells and the inhibition of p38 MAPK and PKCζ repressed the growth effect caused by GLP-1, suggesting the involvement of these two pathways in GLP-1-mediated proliferative effect (46). Heller *et al*., suggested a new mechanism for the action of GLP-1 and exendin-4 in rat pancreatic cell lines via the up-regulation of wnt-4 which is a novel regulator of beta cell proliferation (61). In the same rat cell model, GLP-1 was shown to inhibit SirT1 which was identified as a negative regulator of beta cell proliferation (45). Another mechanism by which GLP-1 analogues positively regulate beta cell mass is through increasing the level of adiponectin and down-regulating C-reactive protein expression (62). In addition, GLP-1 induced cyclin D expression via the cAMP/protein kinase A (PKA) pathway (43).

Since diabetic patients exhibit glucolipotoxicity and beta cell apoptosis, several studies have investigated the role of GLP-1 and its analogues in reversing these adverse effects *in vitro*. GLP-1 inhibited apoptosis after serum starvation in insulin producing MIN6 cells by mediating signaling through the PI3k/PKA pathways (63) and prevented methylglyoxal-induced apoptosis in rat cells by improving mitochondrial function and inhibition of AMPK activation (64). Exendin-4 protected rat ins-1 cell lines from glucolipotoxicity-induced ER stress through down-regulation

of SREBP1c (65) and reversed the effects of cytokines on mitochondrial proteins (66). In addition, exendin-4 inhibited oxidative stress in rat cells by activating nuclear factor erythroid 2-related factor 2 (Nrf2) (67) and promoted the survival of mouse MIN6 cells in lipotoxic conditions via the ERK1/2 pathway (68). Liraglutide improved rat beta cell survival via the AMPK/mTOR/P70S6K signaling pathway and protected the cells against glucolipotoxicity by activating mTOR (48) and through down-regulation of micro RNA miR-139-5p in diabetic rat *in vivo* and the rat ins-1 cell line (69). Figure 3 summarizes some of the pathways involved in rat pancreatic cell proliferation and apoptosis inhibition after GLP-1R activation (49).

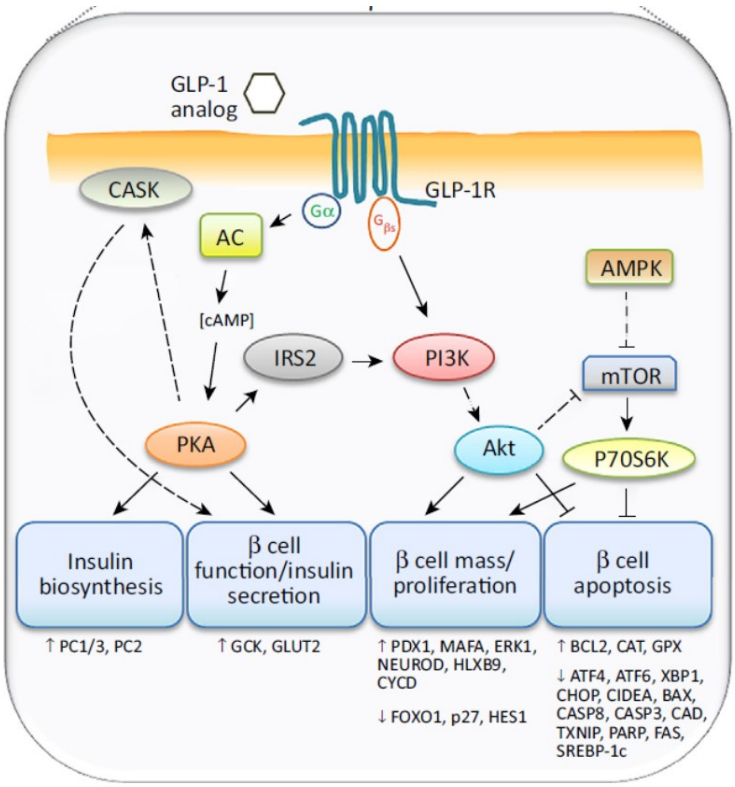

Figure 3: Pathways activated upon binding of the GLP-1 or GLP-1R agonists to the GLP-1R on pancreatic beta cells. PDX1: pancreas/duodenum homeobox protein 1. MAFA: transcription factor MafA. NEUROD: neurogenic differentiation factor 1. HLXB9: motor neuron and pancreas homeobox protein 1. FOXO1: forkhead box protein O1. P27: cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$). HES1: transcription factor HES-1. Bcl2: apoptosis regulator Bcl-2. CAT: cationic amino acid transporter 2. GPX: glutathione peroxidase 1. ATF4: cyclic AMP-dependent transcription factor ATF-4. ATF6: cyclic AMP-dependent transcription factor ATF-6 alpha. XBP1: X-box-binding protein 1. CHOP: DNA damage-inducible transcript 3 protein (Ddit3). CIDEA: cell death activator CIDE-A. Bax: apoptosis regulator BAX. CASP8: caspase 8. CASP3: caspase 3. CAD: CAD protein. TXNIP: thioredoxin-interacting protein. PARP:

poly [ADP-ribose] polymerase 1. FAS: tumor necrosis factor receptor superfamily member 6. SREBP-c: sterol regulatory element-binding protein 1.

The above studies were conducted mainly on rat ins-1 cells and mouse and rat islets. GLP-1 and its analogues show anti-apoptotic and proliferative effects on human islet beta cells as well. GLP-1 reduced apoptosis in cultured human islets by up-regulating the anti-apoptotic Bcl-2 protein and down-regulating caspase 3 (70). Liraglutide treatment enhanced human islets survival rate *in vitro* and their proliferation capacity, though the latter effect was insignificant (71). In addition, liraglutide induced proliferation of human non-sorted beta cells *in vitro* when cultured on bovine corneal endothelial cell matrix, while no effect was seen on the sorted beta cells (72), probably reflecting the fact that cell sorting affects the viability and functionality of cells when re-cultured. Exendin-4 was able to induce replication of human islets from young donors (< 25 years of age) but not older donors (> 35 years of age) *in vitro*. This replication was also translated *in vivo* when the islets were engrafted in mice (73). Park and colleagues hypothesized that exenatide might induce human beta cell proliferation via the Irs2/PI3k/Akt pathway since it increased Irs2 expression and stimulated Akt (protein kinase B) phosphorylation in these islets (74). In a proteomic study, GLP-1 protected human islets against cytokine-induced apoptosis (75).

The previously mentioned studies were mostly targeted, identifying potential pathways involved in GLP-1 mediated proliferation or survival. Proteomics studies on the effect of GLP-1 and its analogues are limited. Tews *et al* were able to show that exendin-4 reduced oxidative stress induced by cytokines by performing proteomic analysis of the isolated mitochondria of rat ins-1 cells (66). Kim *et al* identified the 14-3-3 protein family to be involved in the protective effect of exenatide against streptozotocin and palmitate by blocking their posttranslational modification and restoring the protein level of 14-3-3θ (76,77). A proteomic study on human islets highlighted the protective role of GLP-1 treatment in cytokine mediated cell stress through the up-regulation of cytoskeletal, metabolism and islets regeneration proteins (75). Based on what preceded, we aimed to fill the gap in the proteomics studies, highlighting the changes after exendin-4 treatment in beta cells using chemical labeling approaches and phosphoproteomic enrichment followed by Mass Spectrometry analysis. In this respect, we will

provide a brief background to the field of Proteomics and Mass Spectrometric analysis in the following section.

III. Proteomics and Mass Spectrometry

A. Introduction

Proteomics is the identification of all proteins present in a cell or organism, which is known as the proteome. The proteome is the whole set of proteins expressed in a cell or organism at a specific time, under a specific condition. It comprises two categories: the expression proteome, which is a representation of the proteins expressed in the biological system, and the functional proteome, which is a characterization of protein interactions that translates into pathway analysis (78). A more thorough definition of proteomics was given by Fields in 2001: "Proteomics includes not only the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities, and, ultimately, their function" (79). Currently, proteomic analysis accounts for 19% of biomarker discovery (80).

Mass spectrometry (MS) is a highly sensitive technique the beginnings of which date back to the early 1900s, yet its role in biological sciences only became pronounced in the 1980s and 1990s (81) due to the rise of soft ionization techniques compatible with biological samples. In general, mass spectrometers employ an ion source, a mass analyzer and a detector. In the context of proteomics, the underlying principles are ionization of proteins or peptides (enzyme-digested proteins), followed by determination of the mass to charge ratio (m/z) of the analytes in the gas phase as well as isolation of individual analytes, fragmentation and capturing of the m/z spectrum of the resulting products. This acquisition scheme combining precursor and fragment ion spectra is commonly known as tandem mass spectrometry, MS/MS or MS^2. The produced data on precursor ion mass and fragmentation spectra are most commonly used for *in silico* matching against organism-specific protein databases (82).

As mentioned above, peptides have to enter the mass spectrometer as ions. In this respect, there are two types of biocompatible ionizations: electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), both of which were discovered in the 1980s (83) and acknowledged in 2002 by awarding a Nobel Prize to the developers. ESI is most widely used because it ionizes peptides in the liquid state, generates stable ions and is it not affected by the mass of the peptide prior to ionization (84). The principle of this technique is that, mediated by

high electric fields and chromatography, analytes in acidic or basic solution form small droplets which are desolvated by heat and vacuum exposure and through repulsion between charged analytes finally result in ionization into the gas phase (84). MALDI ionizes and transfers analytes into the gas phase by using LASER pulses to liberate them from co-crystals with a laser energy absorbing matrix (83).

Two main types of proteomic analysis by mass spectrometry exist: bottom-up and top-down proteomics. The bottom-up proteomics, also referred to as shotgun proteomics, is the most commonly used technique and relies on the principle that complex protein mixtures are digested into peptides using proteases (mainly trypsin). These peptides are then injected, ionized and analyzed as mentioned above. Top-down proteomics is based on the principle of injecting intact proteins into the mass spectrometer. While this approach is fraught with difficulty due, for example, to reduced ionizability and analyte-species multiplication by mass to charge state and post-translational modifications (PTM), it offers the benefit of not having to assemble protein-level information from peptide data and can detect PTMs in their combinatorial context, as well as characterize protein isoforms. Due to its limitations, the technique is mainly applied for analysis of single proteins or simple protein complexes (85). What follows will focus on the bottom-up approach of proteomic analysis. Figure 4 summarizes the steps of mass spectrometrical analysis.

Due to the complexity of biological materials and the wide dynamic range of expression level of proteins in a cell, fractionation techniques were introduced and applied prior to sample injection into the mass spectrometer. The most widely used approach is fractionating peptides based on their hydrophobicity using a reverse phase liquid chromatography (LC) column (known as online separation) coupled to a mass spectrometer. For analysis of large numbers of peptides, other fractionation techniques are applied prior to loading the peptides on the LC column (known as offline separation). Such separation can for example be done based on size (using gel electrophoresis or size exclusion chromatography columns) or isoelectric point (using immobilized pH gradient gel strips or ion exchange chromatography columns) (86).

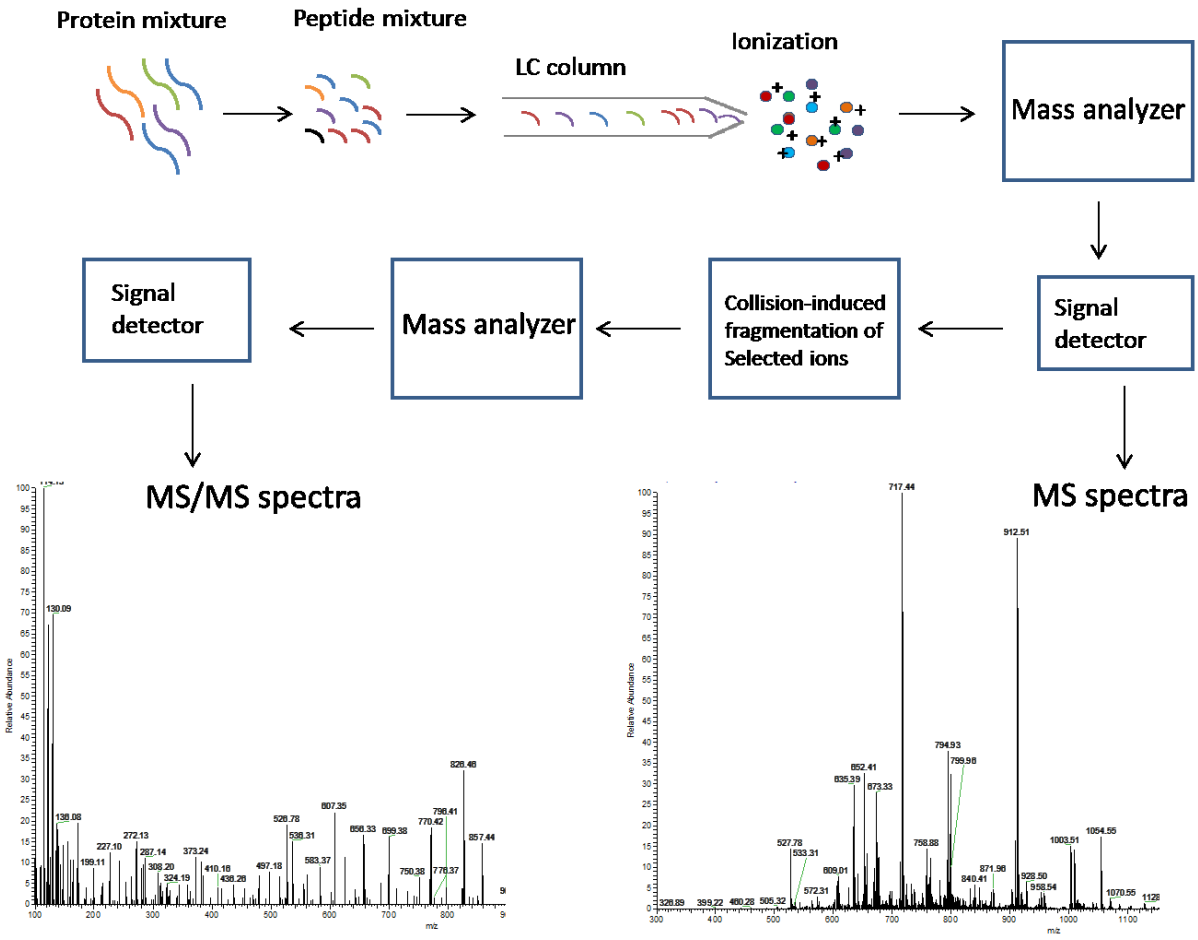

Figure 4: Steps required for mass spectrometry analysis. The procedure starts with peptide digestion and finishes by obtaining the MS/MS spectra. In the spectra images, the x-axis shows the m/z values while the y-axis displays the relative abundance of the peptides.

B. Quantitative proteomics

Quantitation in proteomics stems from the principle of comparing peptides or proteins, either from the same experimental preparation or from different experiments. Quantitation can be absolute or relative. The latter concludes whether a specific protein is over-expressed or under-expressed in a sample compared to another, while the absolute quantitation gives the exact amount of a protein in a mixture as compared to a spiked-in standard of known concentration (87). Mass spectrometry is, due to the inherent dynamic range limitations, charge competition and other effects, semi-quantitative at best. None the less, direct comparison between ion intensities in parallel runs may be used for comparative quantitation. Such strategies are referred to as “label free” approaches. Overcoming the limitations referred to above at the

expense of more involved sample processing, isotopic labeling of peptides is often employed to create a mass shift that can be detected and reported. Relative quantitation using stable isotopic labeling is the most used approach and is the method of choice in our study.

i. Label-based quantitation

This type of quantitation introduces a stable isotopic label on specific amino acids of a protein therefore changing its mass and consequently its m/z ratio and allows for its comparison with a differentially labeled counterpart. Differentially labeled peptides or proteins are mixed together in a 1:1 ratio and injected as one sample into the mass spectrometer. There are three types of labels: chemical, metabolic and enzymatic (87). Chemical labeling is based on adding a label to proteins or peptides through a chemical reaction between the reagent and the sample. Several types of chemical labeling exist such as the dimethyl labeling which utilizes different masses of formaldehyde and cyanoborhydride to introduce light, intermediate and heavy dimethyl labels on lysine residues (88). Samples to be mixed are first digested into peptides, chemically labeled and then mixed 1:1 after ensuring labeling efficiency. Metabolic labeling is introducing a label early in the experiment, during cell culture for instance, where light, medium or heavy forms of amino acids are added into the culture media and allowed to incorporate as the cells grow (89). This labeling introduces fewer errors into the sample processing since samples can be mixed before peptide digestion either at the protein level after quantification or at the cellular level after cell counting. Enzymatic labeling occurs when the protein digestion into peptides takes place in heavy (H 2 18O) or light (H 2 16O) water. This reaction introduces two ^{18}O or ^{16}O atoms at the C terminus of the generated peptides, respectively (90).

ii. Label-free quantitation

Label-free quantitation is a much simpler and straightforward technique as it requires fewer preparation steps before the samples are analyzed. The samples to be compared are run independently on the machine and the comparisons done are based on either spectral peak intensity or the number of observations of a peptide, which reflects its abundance (87). However the shortcomings of this technique are the need for double the measuring time and

the run-to-run variation which compromises the result accuracy. For instance, if a very intense peak elutes in one condition, but not the other, the signal will drop resulting in intensity variation between the two runs. Figure 5 represents the differences between the types of labels and the label-free approach (91).

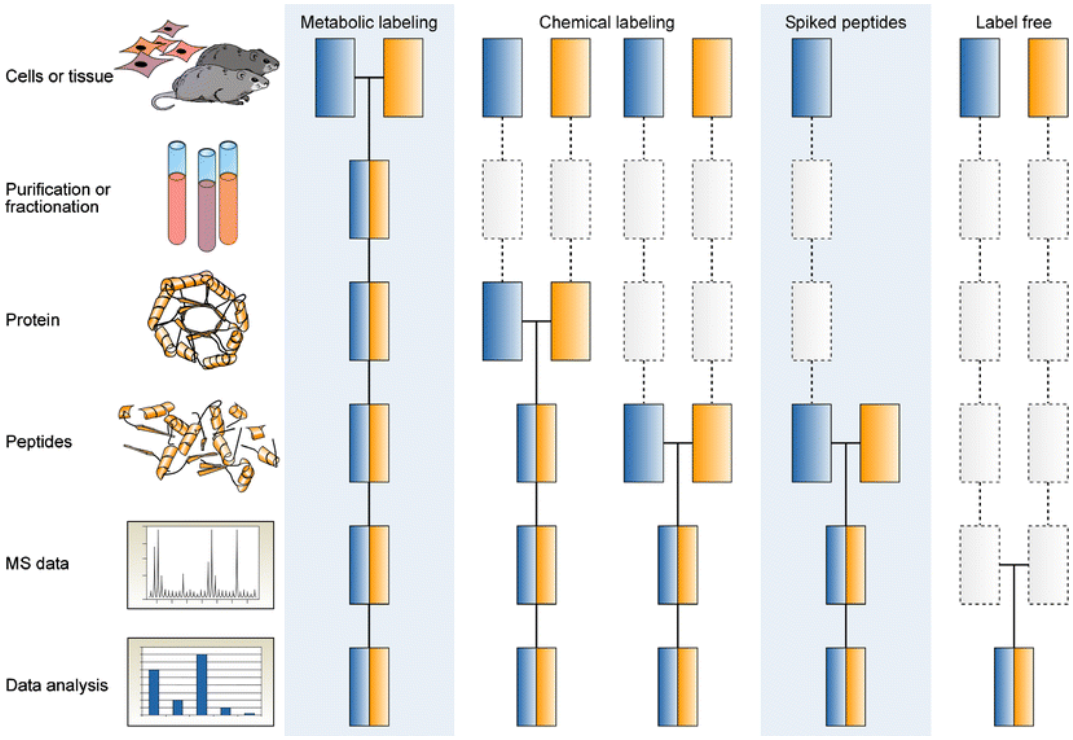

Figure 5: Labeling workflows applied in quantitative proteomics. Dashed lines represent the point of the experiment where error can occur. Blue and orange represent differently labeled samples. Horizontal lines represent where the mixing of the differently labeled samples occurs. Errors occur when differently labeled samples are processed individually.

C. Post translational modifications (PTM)

PTM is the natural process of altering a protein through adding a functional group to one of its amino acids. It is a crucial step in cellular processes because it may alter the function of the protein. Thus, identification and characterization of these PTMs are important for understanding many biological processes in health or disease states such as cancer (92). There are more than 200 physiologically relevant PTMs known to occur in a cell (93) few of which have, however, been extensively studied. Among these are phosphorylation, acetylation,

glycosylation, methylation, ubiquitination, and sumoylation (93). Since modified proteins constitute a subset of the whole proteome, enrichment strategies have been applied to isolate these proteins so they can be analyzed separately. Examples for such strategies include the interaction of titanium dioxide beads with a phosphate group and the hydrophilic interaction liquid chromatography (HILIC) for glycosylated peptides (94). The enriched modified proteins still form a complex mixture that requires fractionation prior to analysis by MS.

D. Phosphoproteomics

Protein phosphorylation is a commonly studied PTM since it plays a major role in diverse processes such as cell cycle progression, signal transduction and apoptosis. Perturbations to the phosphorylation state can lead to disease development (95). Phosphorylation naturally occurs most commonly on serine, threonine and tyrosine residues. Phosphorylation could also occur on other residues including histidine, lysine, arginine, cysteine, asparagine and glutamine, but is usually lost under acidic conditions rendering the study of the phosphorylation of these residues difficult using conventional methodologies (96). While the biological significance of phosphorylation at these residues remains to be uncovered, phosphohistidine and phosphocysteine are involved in cellular signaling in prokaryotes (97,98). In addition, phospholysine and phosphohistidine have been identified in rat and bovine liver cells, respectively; however the biological functionality of this modification was not characterized (96).

Advances in MS techniques have led to the wide ranging identification of phosphopeptides especially from shotgun proteomics (99). Phosphosite databases like KinaseNet (100) and PhosphoSitePlus (101) have been established where the latter reports around 169000 phospho-serine, 70000 phospho-threonine and 44000 phospho-tyrosine sites across species (101). An early global phosphoproteomic study in HeLa cells was able to identify 2244 phosphoproteins and 6600 phosphopeptides in the EGF signaling cascade (102) and these studies were extended to identify 50,000 unique phosphopeptides corresponding to 38,229 phosphorylation events on 7,832 proteins from 6 mg of protein input material (103). In addition, mapping of 23,415 phosphosites into 14 rat tissues was done in a study in 2012 (104).

A key step in MS-based phosphoproteomic analysis is sample processing. Phosphate groups are labile and readily cleaved by phosphatases once the cells are lysed. Therefore, the addition of phosphatase inhibitors in the sample preparation is crucial for the identification of phosphopeptides. Quantitative phosphoproteomics employs the same steps in sample preparation and MS analysis as quantitative proteomics, with the addition of phosphopeptide enrichment. Two phosphopeptide enrichment techniques are currently widely used : metal oxide affinity chromatography (MOAC) and immobilized metal ion affinity chromatography (IMAC) (94), however the combination of both steps in one protocol with some adaptation in the elution and fractionation steps allowed for the identification of ~ 6600 unique phosphopeptides, including and mono- and multi-phosphorylated, from just 300 µg of proteins, which is considered a low amount of input material in comparison to other studies (105). The principle of both techniques is based on the adsorption of the negatively charged phosphate groups to positively charged titanium or IMAC beads followed by sequential elution and fractionation of the phosphopeptides.

Quantification of phosphopeptides and sites is done using specific algorithms such as MaxQuant which searches the peptide spectra against established databases and in the process maps the phosphopeptides with the phosphorylated amino acid to a specific protein. However, a key limitation in quantitative phosphoproteomics is the false interpretation of phosphosite abundance changes resulting from changes in protein expression and not really reflecting an increase or decrease of phospho-occupancy. In this study we thus correct for protein abundance as previously proposed by others (106). The correction for protein abundance reveals the phosphosite occupancy which, in our opinion, holds the most significant biological information.

IV. Cell Lines

As discussed previously, studies on induction of beta cell proliferation are mainly done *in vitro*, utilizing either isolated islets or cell lines. Among the most studied cell lines are the rat ins-1 and its derivative ins-832/13. The ins-1 cell line was isolated in 1992 by Asfari and his colleagues from a rat insulinoma. The cells were gamma irradiated and remained glucose responsive in culture for several passages (107). The cell line has been comprehensively studied since then as it shows some beta cell characteristics such as glucose responsiveness in addition to its expression of the GLP-1 receptor which allowed for extensive studies of the pathways involved after treatment with that peptide. Ins832-13 was established in 2000 through the stable transfection of the ins-1 cells with the human insulin gene (108). The cells were highly glucose responsive with increased growth rate and were suggested for metabolic signaling studies (108).

Human beta cell lines have been recently developed and applied in glucose responsiveness studies. The 1.1B4 human cell line was established by electrofusion of human beta cells with the human ductal epithelial pancreatic cancer PANC-1 cells (109). The cells proved to be glucose responsive and to express the GLP-1 receptor (110). However no studies on the proliferative effects of GLP-1 and its analogues have been reported yet in these cells. In 2011, another human beta cell line was developed by group in France through lentiviral vector transfection of human fetal pancreatic tissue (111). The cells were glucose responsive and expressed several beta cell markers; however, they continuously proliferated. The cell line was further engineered to generate conditionally immortalized human β cell line based on excision of the immortalizing transgenes (112).

We were able to acquire the rat ins-1 and ins-832 cells lines and the human 1.1B4 line to validate their reported glucose responsiveness and GLP-1 effects (for the rat cells lines), test the effect of GLP-1 analogue, exendin-4 on the growth of the 1.1B4 cells and perform the proteomic and phosphoproteomic screen on the selected cell line.

Hypothesis and Aims

Based on the background discussed above, we hypothesize that several proteins downstream of GLP-1 may represent potential targets for the treatment of diabetes using small molecules.

We therefore aim to:

1. Validate the candidacy of commercially available cell lines for performing our proteomic and phosphoproteomic screen
2. Characterize the proteins and signaling pathway(s) involved with exendin-4 induced beta cell proliferation using proteomic and phosphoproteomic analysis

Materials and Methods

I. 1.1B4 cells

A. Cell Culture

Human pancreatic cells, 1.1B4 (Sigma, Germany), were cultured in complete RPMI media (Gibco Life Technologies, USA) supplemented with 10% fetal bovine serum (GE Healthcare Hyclone, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Corning, USA). Cells were maintained in a humidified incubator at 37°C and 5% CO2.

B. Insulin ELISA

Cells were seeded at a density of 200,000 cells per well in a 24-well plate. When the cells reached 100% confluence, glucose stimulated insulin secretion (GSIS) was performed in Krebs-Ringer buffer (116 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.19 mM KH_2PO_4, 23.96 mM $NaHCO_3$, 10 mM HEPES, 0.2% BSA) for 1 hour at basal glucose levels, followed by 45 minutes of KRB buffer with different secretagogues, including Exendin-4 (Bachem, Switzerland). The glucose concentrations used are based on previous studies on these cell lines (110). The supernatant was collected, spun at 10000xg for 3 min to remove floating cells, aliquoted and stored at -20°C. The Mercodia Ultrasensitive Rat Insulin ELISA kit (Mercodia, Sweden) was used according to the manufacturer's protocol. The absorbance was read at 450nm using CLARIOstar automated plate reader (BMG LABTECH, Germany). This experiment was done in three replicates with each condition comprising three wells.

C. Bromodeoxyuridine (Brdu) incorporation assay

Brdu incorporation was checked using the BrdU Cell Proliferation ELISA colorimetric kit (Abcam, United Kingdom). Briefly, cells were seeded in 96-well plates and starved in serum free media containing 3 mM glucose and 0.1% bovine serum albumin (BSA) for 24 hours. Cells were then treated with 10 nM, 50 nM and 100 nM of exendin-4 for 24 hours in starvation media. Brdu was added to the cells at a final concentration of 10 µM and incubated overnight after which the cells were washed, fixed and incubated with the corresponding primary and secondary

antibodies. After adding the peroxidase substrate, the plate was incubated in the dark and absorbance was read at 450 nm using CLARIOstar automated plate reader (BMG LABTECH, Germany). This experiment was done in three replicates with each condition comprising four wells.

II. Ins-1 and Ins832-13 rat cells

A. Cell Culture

Rat ins-1 and ins832-13 cells were a kind gift from Dr. Christopher Newgard (Duke University, North Carolina) and were grown in RPMI 1640 (Gibco, Life Technologies, USA), containing 2 mM L-glutamine, 10 mM HEPES (Gibco, Life Technologies, USA), 10% dialyzed fetal bovine serum (GE Healthcare HyClone,) 100 U/ml penicillin G, 100 µg/ml streptomycin (Corning, USA), 1 mM sodium pyruvate (Gibco, Life Technologies, USA) and 50 µM 2-mercaptoethanol (Sigma, Germany). Cells were maintained in a humidified incubator at 37°C and 5% CO2.

B. Insulin ELISA

Ins832-13 cells were seeded at a density of 200,000 cells/well in a 24-well plate. After three days, glucose stimulated insulin secretion (GSIS) was performed in Krebs-Ringer buffer (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.1 6 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, and 0.2% bovine serum albumin) as previously described (108). Cells were incubated in the buffer at basal glucose levels for 1.5 hours followed by 30 minutes of KRB buffer with 15mM glucose and 10nM exendin-4. The supernatant was collected and spun for 3 min at 10000xg to precipitate any floating cells and was aliquoted and stored at -20°C for analysis. The Mercodia Ultrasensitive Rat Insulin ELISA kit (Mercodia, Sweden) was used according to the manufacturer's protocol. This experiment was done in three replicates with each condition comprising three wells.

For the ins-1 cells, the Insulin (Rat) High Range ELISA kit (Alpco, USA) was used. The cells were seeded at a density of 100000 cells per well in a 24-well plate and GSIS was performed after 3 days using the same protocol as ins832-13 cells. The absorbance was read at 450nm using

CLARIOstar automated plate reader (BMG LABTECH, Germany). This experiment was done in three replicates with each condition comprising three wells.

C. Bromodeoxyuridine (Brdu) Incorporation Assay

Brdu incorporation was checked using the Cell Proliferation ELISA, Brdu (colorimetric) kit (Roche, USA). The reason we changed to this kit is because it has been used in many publications and recommended by practitioners at our institute for its time saving protocol and sensitivity. The assay was performed according to the manufacturer's protocol. Briefly, cells were cultured at a density of 50000 cells/well for ins832-13 cells and 80000 cells/well for ins-1 in a 96-well plate and kept in culture for 2 days. Cells were then starved in serum free media containing 3 mM glucose and 0.1% BSA for 24hrs then treated with 100 nM exendin-4 for another 24 hours in starvation media. Brdu was added to the cells at a final concentration of 10 µM for the last 4 hours. Cells were then fixed and the antibody added for 50 minutes after which the cells were washed and the substrate added for 30 minutes. The plate was read at 450nm and 690nm using CLARIOstar automated plate reader (BMG LABTECH, Germany). This experiment was done in three replicates with each condition comprising three wells.

D. Protein Extraction for Western Blot

Cells were seeded in T25 flasks until 80% confluence and were starved overnight in serum free media containing 3 mM glucose and 0.1% BSA. Cells were then incubated with 10 nM exendin-4 in starvation media for different time points. Cells were lysed on ice in 0.2% sodium dodecyl sulphate (SDS) (Sigma, Switzerland) with phosphatase inhibitor, (Roche, Germany), protease inhibitor (Roche, Germany) and benzonase (Sigma, Denmark). Proteins were quantified using Quick Start™ Bradford 1x Dye Reagent (Biorad, USA). This experiment was repeated three times.

E. Protein Gel Electrophoresis and Western Blot

Prior to running the gel, 35 µg of extracted proteins were mixed with 100 mM dithiothreitol (DTT) (Sigma) and NuPAGE® LDS Sample Buffer (4X) (ThermoFisher, USA) and incubated at 95°C

for 5 minutes. NuPAGE™ Novex™ 4-12% Bis-Tris gels from Invitrogen were used. The gels were run in MES SDS Running Buffer (G-biosciences) under the following conditions: 200 volt and 125 mA for 45 minutes. The gels were rinsed in water and blotted onto nitrocellulose membranes (GE Healthcare, Germany) in 1X transfer buffer (tris base, glycine and 10% methanol, Sigma) for 2 hours at 15 volts and 140 mAmp. The membranes were then washed with 1X tris-buffered saline (TBS) (tris base and sodium chloride, Sigma), blocked for 1 hour in 5% non-fat milk in 1XTBS and incubated overnight with pAkt-ser473 (Cell Signaling, USA) and total Akt (Cell Signaling, USA) antibodies diluted 1:1000 in 5% BSA (Sigma, USA) in 1XTBS with 0.1% tween (TBS-T). The following day the membrane was washed 3 times in 1XTBS after which a fluorescently labeled secondary antibody (Jackson Laboratory, USA) was added for 1 hour at a concentration of 1 in 10000 in 5% non-fat milk in 1XTBS-T. GAPDH antibody (Cell Signaling, USA) was used as a loading control and incubated for 1 hour at a concentration of 1:1000 in 5% non-fat milk in 1XTBS-T. The membrane was exposed in a LICOR ODYSSEY CLx IR Imaging machine (LI COR Biosciences, USA). This experiment was repeated three times.

III. Proteome and phosphoproteome enrichment and analysis

A. Protein Extraction for proteomic analysis

Ins-1 cells were seeded in T75 flasks and were starved in serum free media with 3mM glucose and 0.1% BSA for 24 hours then treated with 100nM exendin-4 for another 24 hours in starvation media. Control cells were treated with 1XPBS (phosphate buffer saline). Cells were then lysed on ice in 0.2% SDS (Sigma, Switzerland) with phosphatase and protease inhibitors (Roche, Switzerland) and benzonase (Sigma, Denmark). The proteins were sonicated for 3 times, 3 seconds each, and centrifuged at 10000xg for 10 minutes. The proteins were then precipitated in methanol and chloroform (Sigma, USA) as previously described (113) and resuspended in 2 M of urea/thiourea (Sigma, Germany) in 100 mM triethylammonium biocarbonate (TEAB) (Sigma, Switzerland). The protein concentration was determined using Quick Start™ Bradford 1x Dye Reagent (Biorad, USA). This experiment was repeated three times and the downstream techniques discussed below were performed for each replicate.

B. In-solution Digest

After 30 minutes reduction with 10 mM of dithiothreitol (DTT) and 20 minutes alkylation with 20 mM iodoacetamide (Sigma, USA) in the dark, proteins were digested using endoproteinase Lys-C (Wako Chemicals, USA) for 3 hours at room temperature. Proteins were then digested by trypsin (Promega, USA) overnight at room temperature. Lys-C and trypsin were used at an enzyme to protein ratio of 1:50. The digested peptides were kept in – 80°C until further use.

C. Reductive Dimethyl Labeling of Peptides

Stable isotope dimethyl labeling was performed on peptides based on a previously established protocol (88). Briefly, peptides of exendin-4 treated samples were labeled as "heavy" and the untreated control were labeled as "light". Light form of formaldehyde (4% CH_2O in H_2O) (Sigma, Netherlands) and sodium cyanoborohydride (0.6 M of $NaBH_3CN$ in H_2O) (Sigma, USA) were added to the control peptides as the light label. The heavy form of formaldehyde (4% $13CD_2O$ in D_2O) (Sigma, USA) and sodium cyanoborodeuteride (0.6 M of $NaBD_3CN$ in D2O) (Santa Cruz, USA) were added to peptides from exendin-4 treated cells as the heavy label. After incubation at 22 °C for 1 hour with gentle mixing, the reactions were quenched by adding 1% of ammonia solution (Millipore Chemicals, Germany) followed by 5% of formic acid (Fluka, USA). The two differently labeled samples were mixed at a ratio of 1:1 and stored at -80°C before cleaning by removing salts.

The peptide mixture (250 µg of control and 250 µg of exendin-4 treated) was desalted (cleaned) using Oligo R2 and R3 beads (Applied Biosystems, USA). The column was prepared using a C18 plug (Solid phase extraction disk, Empore, USA) and adding R2/R3 slurry for a length of 2.5cm. The column was conditioned using 100 µl of 0.1% of trifluoroacetic acid (TFA) (Sigma, USA). The peptides were then loaded and another washing step was done with 100 µl of 0.1% TFA after which the peptides were eluted using 60% acetonitrile (ACN) (Fluka, Germany), 0.5% TFA. Eluted peptides were dried down by vacuum centrifugation prior to fractionation by isoelectric focusing and phosphopeptide enrichment.

D. Peptide fractionation by in-solution Isoelectric focusing (IEF)

The dried down labeled peptide mixtures were resuspended in TEAB and 200 µg were separated into 12 fractions using the OFFGEL low resolution kit (Agilent, Germany) on immobilized pH gradient (IPG) gel strips with a pH range of 3-11 (GE Healthcare, Sweden) by isoelectric focusing using an OFFGEL fractionator (Agilent, USA) according to the manufacturer's protocol with some modifications. Glycerol in the buffer was reduced from 6% to 0.3% and the ampholytes from 1% to 0.1%. The peptides in 100 mM TEAB buffer were diluted to 360 µl using HPLC-grade water and mixed with 4 volumes of OFFGEL stock buffer (60 µl OFFGEL Buffer, 300 µl 50% glycerol, ddH20 to 50 ml)(GE Healthcare, Sweden). The strips were placed in the tray, the 12-well frames were placed on the strips and the gel strips were rehydrated with 40 µl of IPG Strip rehydration solution (0.56 ml OFFGEL stock solution, 0.14 ml ddH20) in each well after which 150 µl of sample was added to each well. Mineral oil was applied on pads placed on both sides of the strips and the electrodes placed to make contact with the pads. Focusing settings were as follows: 20 kVh, 4500 V, 50 uA, 200 mW. The peptides were allowed to focus overnight. The separated peptides were transferred to PCR-tubes and the wells washed with 50 µl of 50% methanol, 1% TFA for 15 min. The samples were dried down using vacuum centrifugation. Peptide fractions were stored at −80°C.

E. STAGE (stop-and-go extraction) Tipping

The 12 fractions from the isoelectric focusing were cleaned on STAGE tips before being injected into the mass spectrometer. Three layers of C18 disks (Solid phase extraction disk, Empore, USA) were packed in 200 µl pipette tips and were activated with 200 µl of one methanol wash followed by two washes of 200 µl of 2% acetonitrile/0.1% TFA. After loading, the peptides were washed with 200 µl of 0.5% acetic acid followed by 100 µl of 2% acetonitrile/0.1% TFA as an additional wash to reduce the ampholytes contamination from IPG buffer. Peptides were eluted twice with 20 µl of 60% acetonitrile/0.5% TFA, and then dried down using a vacuum centrifugation. After that, peptides were re-suspended in 0.5% acetic acid to be run in LC-MS/MS or stored in the dry form in -20°C.

F. TiSH enrichment of phosphorylated peptides

Phosphorylated peptides were enriched using a slightly modified Titanium-HILIC-SIMAC (TiSH) protocol (105). Three hundred micrograms of dimethyl labeled peptide mixture were used for enrichment. The sample volume was reduced to 100µl and the volume made up to 1 ml in Loading Buffer: 1 M Glycolic Acid (Fluka, USA), 80% ACN, 5% TFA. The peptides were incubated with 0.6 mg of TiO_2 beads (Titansphere TiO_2 5 µm, GL Science, Japan) per 100 µg of peptides (1.8 mg for 300 µg of peptides) for 10 min on a vortexer (high speed) at room temperature (RT). The supernatant was transferred to a fresh tube with 0.3 mg of beads per 100 µg of peptides and the incubation repeated. The TiO_2 beads were pooled and washed with 100 µl of loading buffer by vortexing for 15s followed by 15s of centrifugation in a table centrifuge at 2000g. The beads were subsequently washed with 100 µl of 80% Acetonitrile/1% TFA and 10% Acetonitrile/0.1% TFA. Beads were dried in a vacuum centrifuge for 5 min and peptides were eluted by incubation with 100 µl of 1% ammonia solution (pH ~ 11.3) (elution buffer) for 10 min on a vortexer. The peptide-beads solution was centrifuged and the elution buffer was passed over a C8 stage tip (Solid phase extraction disk, Empore, USA). The elution step was repeated with 30 µl of elution buffer. Phosphopeptides bound to the C8 plug were eluted with 2 µl of 70% ACN. The eluate was dried down and reconstituted in 200 µl of 50% ACN/2% TFA, by sequentially adding 40 µl 10% TFA, 60 µl of H_2O and 100 µl of 100% ACN.

The enriched peptides were subjected to sequential elution from IMAC (SIMAC) to separate multiphosphorylated and monophosphorylated peptides. The redissolved peptides were incubated with 80 µl of IMAC beads slurry (Sigma, USA) per 300 µg of starting material for 30 min on a vortexer at low speed at RT. Following incubation, the IMAC beads were pelleted by centrifugation for 15s and half of the supernatant was transferred to a low-binding tube. The beads and remaining supernatant were transferred to a 200 µl gel loader tip with a constricted end. The remaining supernatant was separated from the beads by pushing air through the tip using a syringe. The IMAC column was washed slowly with 50 µl of 50% ACN/2% TFA and then 70 µl 20% ACN/2% TFA to elute monophosphorylated peptides. Eighty micoliters of elution buffer was added to the column and the IMAC beads incubated for 5 min. The eluate was collected in a tube and the elution step was repeated. The pooled eluate was acidified with 16

µl of 100% formic acid and 4 µl of 10% TFA and desalted using an R3 stage tip with a plug of C18. The multiphosphorylated peptides were dried down and stored at -80°C for further analysis. The IMAC flow through containing the monophosphorylated peptides was adjusted to 70% ACN/2%TFA and subjected to a second round of TiO_2 enrichment using a single wash step of 50% ACN/0.1% TFA to pool the beads. Following elution, the peptides were dried down for further fractionation on hydrophilic interaction liquid chromatography (HILIC) column.

G. Hydrophilic interaction liquid chromatography

The monophosphorylated peptides were fractionated on an in-house packed column (TSKgel Amide-80 HILIC 5µm) (Tosoh Bioscience, Japan) in a 320 µm×170 mm PEEKsil tubing using the Agilent 1200 HPLC platform (105). Briefly, the monophosphorylated peptides were resuspended in solvent B (90% ACN, 0.1% TFA). The sample was loaded onto the HILIC column and run with a 48 min gradient: 12 µL/min 100% buffer B (90% ACN, 0.1% TFA) for 8.6 min, 8 µL/min 90% buffer B for 0.4 min, 8 µL/min 90%-60% buffer B over 26 min, 8 µL/min 60% -0% buffer B over 4 min, 6 µL/min 100% buffer A (0.1% TFA) for 3 min and 6 min of 100% buffer B to reconstitute the column. Twenty three fractions were automatically collected in a 96 well plate. After drying down and based on the signal intensity provided by the UV detector, fractions were pooled into 8 samples for MS/MS analysis. Figure 6 is a schematic representation of the TiSH protocol.

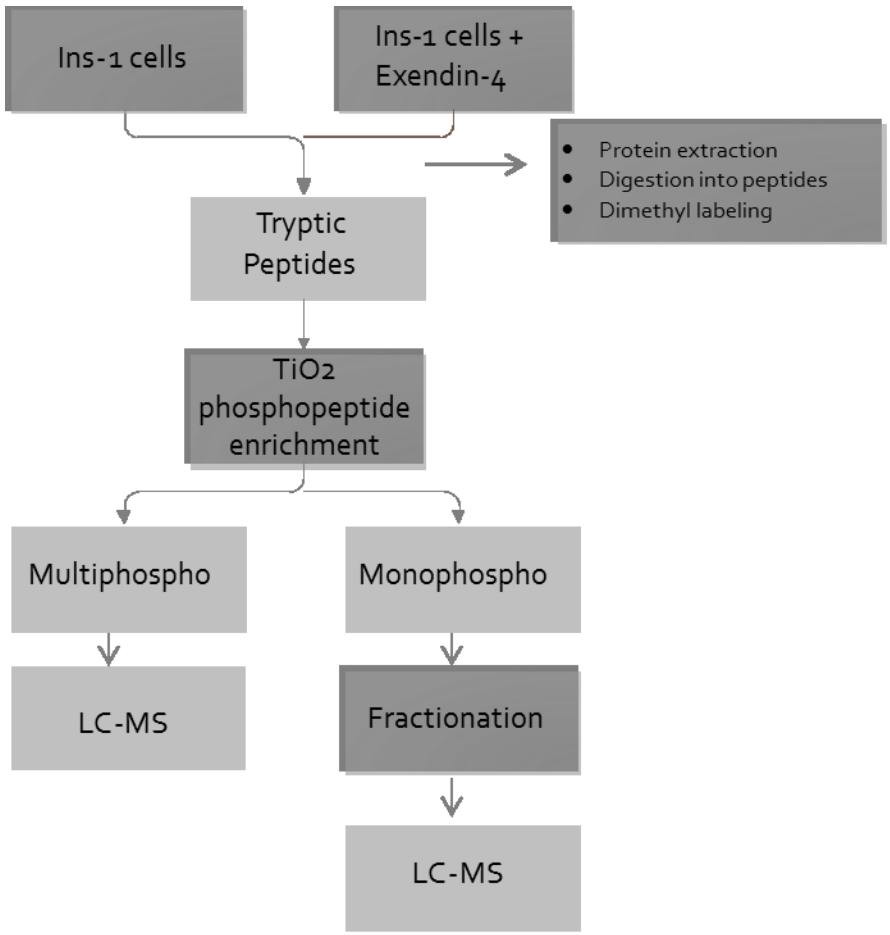

Figure 6: Schematic representation of the TiSH protocol

H. LC-MS/MS

The samples, both from isoelectric focusing and TiSH, were resuspended in buffer A (0.5% formic acid) and run on an Easy-LC coupled to a Q Exactive High Field from Thermo Fisher Scientific. The emitter column was in-house packed with ReproSil-Pur C18 AQ 3 µm beads (Dr. Maisch GmbH, Germany), in an 18 cm and 75 µm ID fused silica capillary (Polymicro Technologies, USA). Two buffers were used in the gradient, buffer A (0.5% formic acid) and buffer B (80% ACN, 0.5% formic acid). Chromatography conditions were defined as follows: H_2O with 0.5% acetic acid for mobile phase A; H_2O: acetonitrile with a ratio of 20:80 (v/v) with 0.5% acetic acid for mobile phase B; flow-rate of 250 ηL/min; injection volume of 6.0 µL and a maximal loading pressure of 280 bars. The gradient length was 120 min with the following profile: buffer B started at 5% and increased to 30% over a period of 95 min, then up to 60%

over a period of 5 min, then from 60% to 95% over a period of 5 min, stayed at 95% for 5 min, then decreased from 95% to 5% over 5 min and remained at this concentration for 5 min.

Precursor scans (MS1 level) were acquired at a resolution of 70000 (at m/z 300), an AGC (advanced gain control) target value of 3e6 charges (maximum ion injection time 20 ms) and m/z scan range of 300 – 1650. Fragmentation spectra (MS2 level) were acquired at a resolution of 17500 (at m/z 300) and an AGC target value of 1e5 charges (maximum ion injection time 120 ms), dynamic exclusion of 25 s and normalized collision energy 25.

I. Data analysis

For the ELISA and WB data analysis, the Mann-Whitney U test was used when two groups were being compared, and the Kruskal-Wallis test followed by Dunn's test post hoc comparisons were used when comparing between more than two groups. $P \leq 0.05$ was considered significant. For the proteomics data, all raw files were searched in one run of MaxQuant version 1.5.6.5, a software package used to analyze raw MS data to identify and quantify peptides and aggregate the results at the protein level. The MS/MS spectra were searched against the Uniprot *Rattus norvegicus* (29795 entries, canonical and isoforms), downloaded on November 14, 2016, using the Andromeda search engine. False discovery rate was set to 1% for peptides, proteins and sites and the minimum peptide length allowed was 7 amino acids. Re-quantify and match between runs were set to true. Multiplicity was set to 2 and labels were light and heavy dimethyl labels (DimethLys0 and DimethNter0 for light; DimethLys8 and DimethNter8 for heavy). Protein quantification was based on two ratio counts. Downstream analysis of the proteomic data was performed using the empirical Bayes moderated T-test implemented by the limma package in an in house R analysis pipeline. P values were corrected for multiple hypothesis testing using the Benjamini-Hochberg correction with an adjusted P value significance cutoff of 5% ($P < 0.05$). Differential expression analysis was calculated based on log2 normalized ratios and proteins with adjusted P value <0.05 were considered significantly differentially expressed. Gene set enrichment analyses were performed using Fisher's exact test with the total dataset as the universe. All functionalities for data analysis downstream of

MaxQuant (normalization, statistics, gene set enrichments based on Gene Ontology) are combined in an in-house-built R package (autonomics).

Results

A. 1.1B4 cells are not glucose responsive and exendin-4 does not significantly enhance insulin secretion.

It was shown by Vasu *et al.* (110) that the human 1.1B4 cells are responsive to glucose and exendin-4 in terms of insulin secretion. To validate these results, we cultured the cells in 24-well plates and allowed them to attach for 3 days. Cells were then incubated with Krebs-Ringer buffer (KRB) containing 1.1 mM glucose and 0.1% BSA for 1 hour to establish basal levels of insulin secretion. The buffer was removed and cells were challenged with a fresh KRB containing 1.1 mM, 5.6 mM and 16.7 mM glucose with 20 nM exendin-4 at the 5.6 mM and 16.7 mM glucose concentrations (Fig.7). The cells were not glucose responsive and the increase in insulin secretion after exendin-4 treatment was not significant.

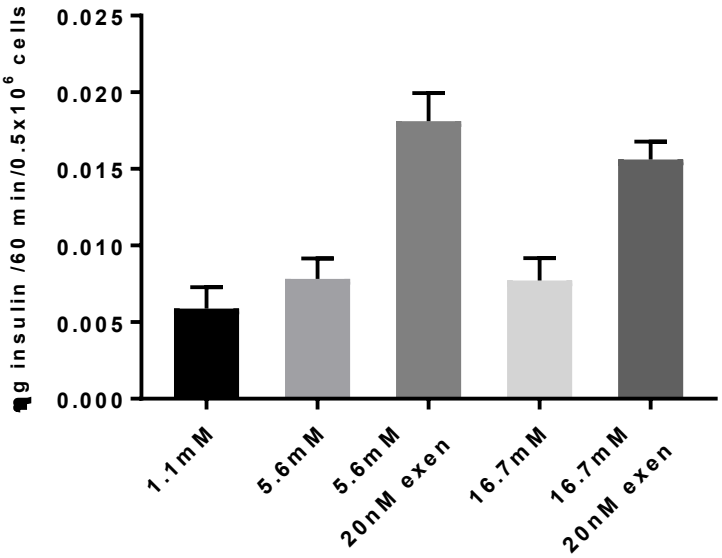

Figure 7: Insulin release from 1.1B4 cells in response to glucose and exendin-4. GSIS was performed for 1 hour in Krebs-Ringer buffer containing 1.1mM glucose and 0.1% BSA followed by another 1 hour-incubation with various glucose concentrations and 20nM exendin-4. Insulin was measured using an ELISA kit from Mercodia and values normalized for cell number. Values are means ± SD of 3 replicates, each comprising 3 wells.

B. Exendin-4 does not induce proliferation in 1.1B4 cells.

Since exendin-4 stimulated insulin secretion, though not significantly, from 1.1B4 cells, despite the lack of glucose sensitivity, we investigated whether this effect translated to proliferation induction. We performed a Brdu incorporation assay. Cells were seeded in 96-well plates and allowed to attach for 3 days. Cells were then starved in serum free media containing 3mM glucose and 0.1% BSA for 24 hours to synchronize them. 10 nM, 50 nM and 100 nM exendin-4 were added to the cells and incubated for 24 hours after which Brdu incorporation was measured using an ELISA. We did not observe any increase in the percentage of Brdu incorporation after treating the cells with various exendin-4 concentrations for 24 hours as shown in figure 8. These data, combined with the lack of glucose and exendin responsiveness in the human cell line, suggested that the 1.1B4 cell line was not an appropriate pancreatic cell model for studying the effect of exendin-4 on proliferation.

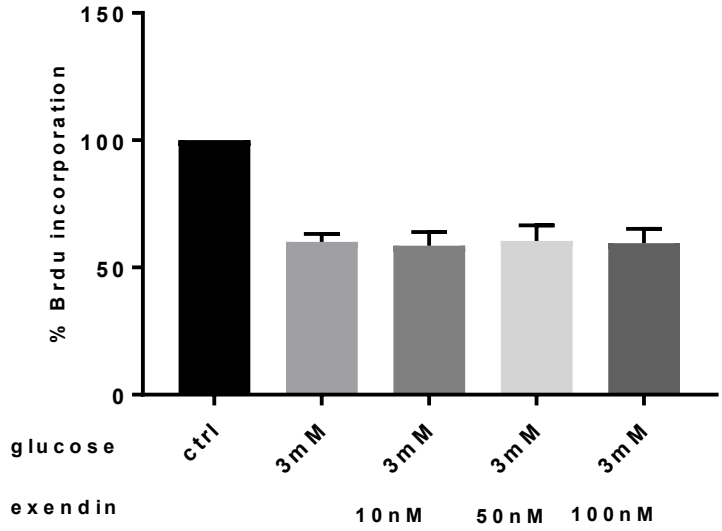

Figure 8: The effect of 24hr exendin-4 treatment on Brdu incorporation in 1.1B4 cells. Cells were serum-starved then treated with various exendin-4 concentrations. Values are means ± SD of 3 replicates, each comprising 4 wells per condition. Values were normalized to the control (resting cells receiving complete growth medium).

C. Exendin-4 and glucose have an additive effect on insulin secretion in rat pancreatic cell lines ins832-13 and ins-1.

Hohmeier *et al.* (108) developed a new cell line derived from the rat parental ins-1 cell line with higher glucose responsiveness. The parental cell line ins-1 and its derivative, ins832-13, were tested to check their glucose and exendin-4 responsiveness. Cells were seeded in 24-well plates and allowed to attach for 3 days. Insulin basal levels were established by incubating the cells for 1.5 hours at 3 mM glucose in KRB. Cells were then challenged with 15 mM glucose or 15mM glucose plus 10nM exendin-4 for 30 minutes. As shown in figures 9 and 10, insulin secretion was higher, though not significantly, following treatment with 15 mM compared to 3 mM glucose. Higher glucose concentration and exendin-4 treatment showed an additive effect on insulin secretion compared to the 3 mM glucose. These results are partially in accordance with the literature suggesting that these cells were an appropriate model for investigating further effects of exendin-4.

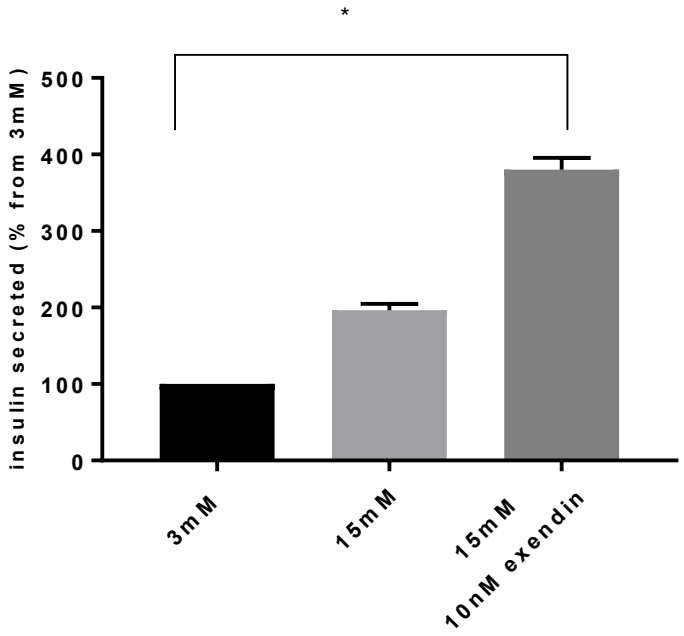

Figure 9: Insulin release from rat ins832-13 cells in response to glucose and exendin-4. GSIS was performed for 1.5 hour in Krebs-Ringer buffer containing 3mM glucose and 0.2% BSA followed by 0.5 hour-incubation with 3mM, 15mM glucose and 10nM exendin-4. Insulin was measured using an ELISA

kit from Mercodia. Values are means ± SD of 3 replicates, each comprising 3 wells. *P value <0.05 for exendin-4 treatment versus 3mM.

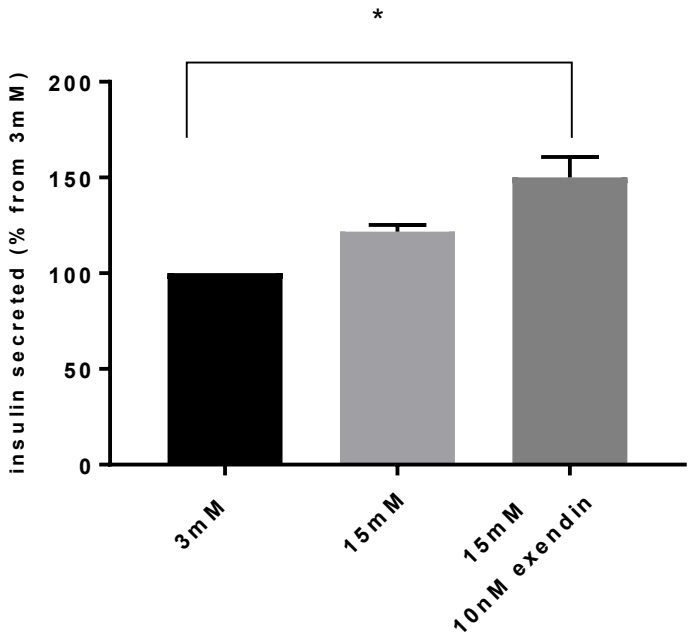

Figure 10: Insulin release from rat ins-1 cells in response to glucose and exendin-4. GSIS was performed for 1.5 hour in Krebs-Ringer buffer containing 3mM glucose and 0.2% BSA followed by 0.5 hour-incubation with 3mM, 15mM glucose and 10nM exendin-4. Insulin was measured using the Alpco High Range Rat ELISA kit. Values are means ± SD of 3 replicates, each comprising 3 wells per condition. *P value <0.05 for exendin-4 treatment versus 3mM.

D. Exendin-4 induces cell proliferation in pancreatic cell lines ins832-13 and ins-1 as shown by Brdu incorporation.

Buteau *et al.* (44,59) have shown proliferative effects of GLP-1 on ins-1 and ins832-13 cells. To confirm this, we seeded the cells in 96-well plates and allowed them to attach for three days. Cells were then starved in serum free media containing 3 mM glucose and 0.1% BSA for 24 hours to synchronize them. Exendin-4 was added to the cells for 24 hours and the results determined by colorimetric assay. As shown in figures 11 and 12, 24 hours exendin-4 treatment induced cell proliferation as demonstrated by the increase in the number of cells that incorporated Brdu. These results suggest that the rat cell lines are appropriate models for further investigation into the effects of exendin-4 on cell proliferation. We went on to use the

parental cell line ins-1 for subsequent studies because it has a slower growth rate and milder insulin response to glucose compared to ins832-13, with both phenotypes being characteristic of pancreatic beta islets.

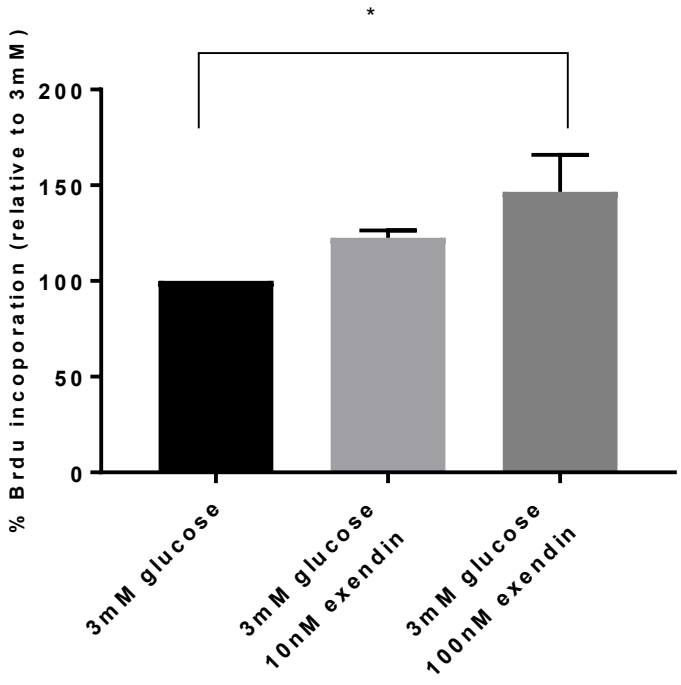

Figure 11: The effect of 24hr exendin-4 treatment on Brdu incorporation in rat ins832-13 cells. Cells were seeded until 80% confluence, starved then treated with various exendin-4 concentrations. Values are means ± SD of 3 replicates, each comprising 4 wells per condition. *P value <0.05 for the 100nM treatment versus 3mM control.

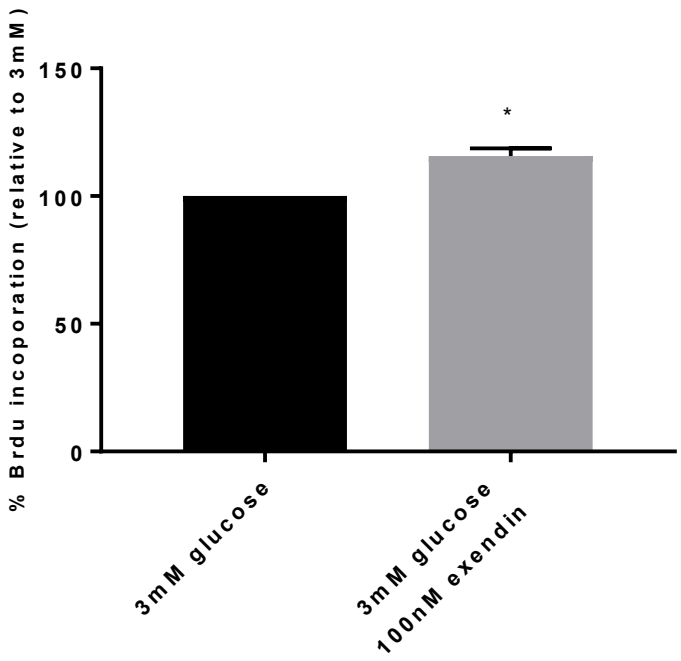

Figure 12: The effect of 24hr exendin-4 treatment on Brdu incorporation in rat ins-1 cells. Cells were seeded until 80% confluence, starved then treated with 100nM exendin-4. Values are means ± SD of 3 replicates, each comprising 3 wells per condition. *P value =0.05.

E. Exendin-4 stimulates protein kinase B (PKB) phosphorylation in ins-1 cells.

GLP-1 was shown to induce PKB phosphorylation in ins-1 cells (60). To validate this in our cells system, cells were seeded in T25 flasks and allowed to attach for 3 days. Cells were then starved overnight in serum-free media containing 3 mM glucose and 0.1% BSA then treated with 10 nM exendin-4 for the specified time points. As shown in figure 13, exendin-4 treatment induced phosphorylation of PKB in a time dependent manner. This result is in accordance with the literature, further confirming that our system could be used as a model for identifying the proteomic and phosphoproteomic changes underlying exendin-4 treatment.

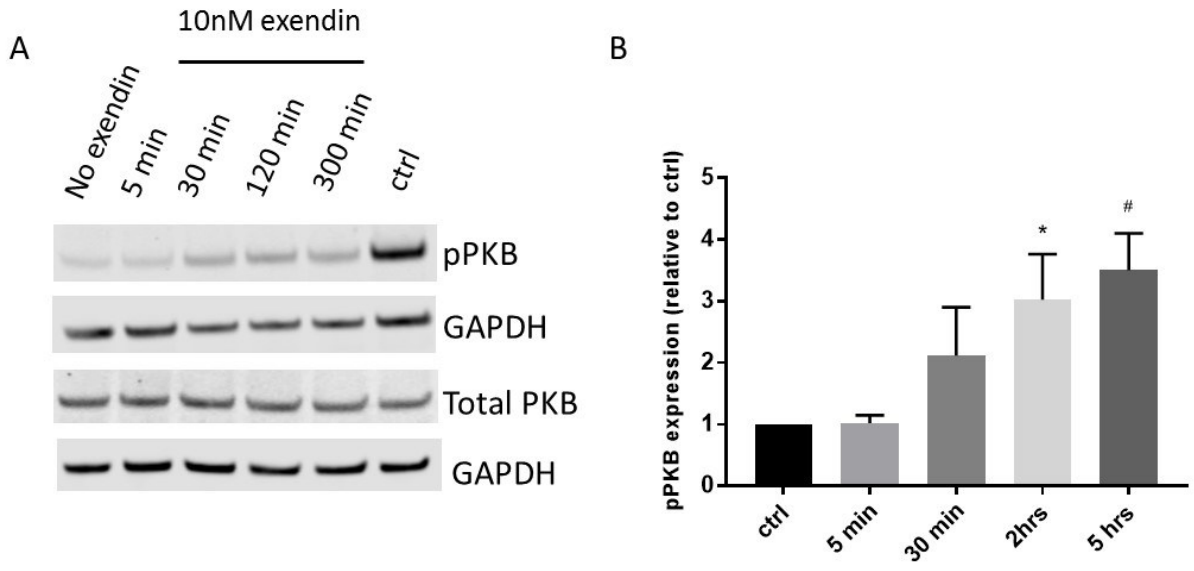

Figure 13: The effect of exendin-4 treatment on the phosphorylation of protein kinase B at ser-473. Cells were seeded in T25 flasks until 80% confluence and were starved overnight in serum fee media containing 3mM glucose and 0.1% BSA. Cells were then incubated with 10nM exendin-4 in starvation media for different time points. A. Representative western blot images. B. Bar graph reflecting the width of the band measured by fluorescence. *P value <0.05 for the 2 hours treatment versus no exendin-4. $^{\#}$P value <0.05 for the 5 hours treatment versus no exendin-4. Results are means ± SD of 3 replicates.

F. Exendin-4 stimulates PKB phosphorylation in ins-1 cells only in the starved state and starvation media does not influence PKB phosphorylation.

Since serum starvation was shown to induce PKB phosphorylation (114), we wanted to show that the phenotype we see is due to exendin-4 treatment and not to the starvation condition. In addition, to choose the best cell culture conditions to use for the proteomics studies, we performed the timed exendin-4 treatment in normal media versus starvation media and checked for the effect on PKB phosphorylation. Cells were seeded in T25 flasks and allowed to attach for 3 days. Cells were then starved overnight in serum-free media containing 3 mM glucose and 0.1% BSA, or kept in complete growth media, then treated with 10 nM exendin-4 for the specified time points. As shown in figure 14A, exendin-4 treatment induced phosphorylation of protein kinase B in a time dependent manner in starvation media but not in complete growth media. This observation suggests that the effect of exendin-4 may be masked

by the effect of insulin and other growth factors in the media and that the starved state is the best condition to study the effect of exendin-4 on the proteome of the rat cell line. To prove that the effect seen on the phosphorylation of PKB is due to exendin-4 treatment and not the result of apoptotic pathways being triggered by the starvation, we incubated the cells as described above in starvation media but without exendin-4. As shown in figure 14B, starvation alone did not stimulate PKB phosphorylation. We concluded that the PKB phosphorylation is driven by the specific effects of exendin-4 treatment and that starvation media was the condition of choice to perform the proteomic and phosphoproteomic study. These experiments were performed once for validation purposes.

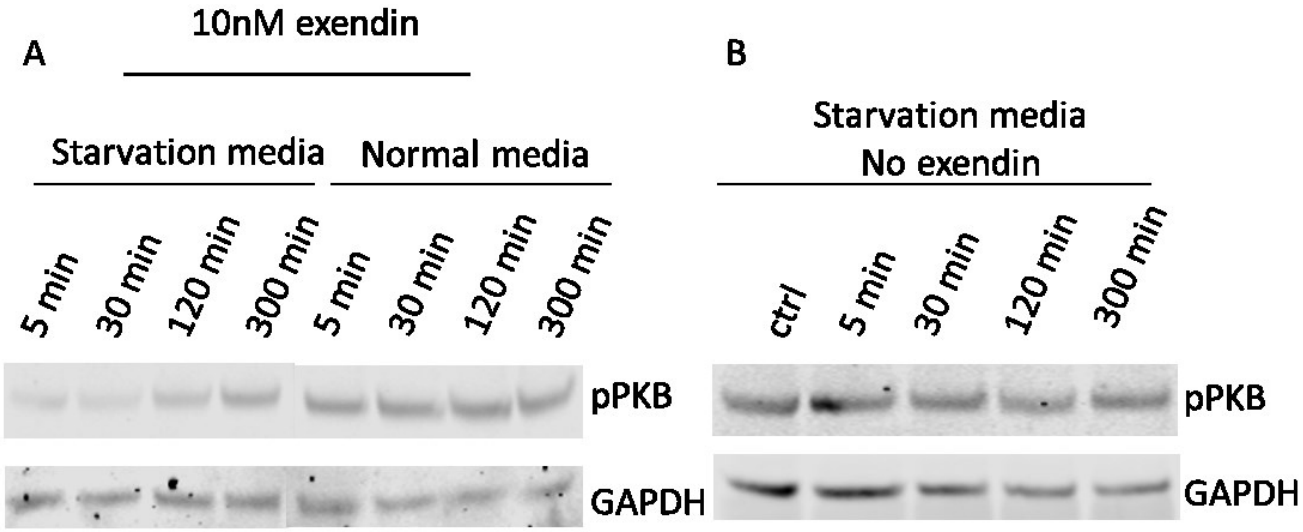

Figure 14: The effect of exendin-4 treatment (in complete and starvation media) and starvation media alone on the phosphorylation of protein kinase B at ser-473. Cells were seeded in T25 flasks until 80% confluence and were A. starved overnight in serum fee media containing 3mM glucose and 0.1% BSA or not starved. Cells were then incubated with 10nM exendin-4 in starvation media or normal media for different time points. B. or kept in starvation media without exendin-4 for different time points.

G. Quantitative proteome analysis in exendin-4 treated versus control cells reveals expression changes in 667 proteins.

Cells were grown in T75 flasks and allowed to attach for 3 days after which they were starved for 24 hours and 100 nM exendin-4 added in starvation media and incubated for further 24 hrs.

Proteins from control and exendin-4 treated cells were extracted, digested into peptides and dimethyl labeled as light for the control and heavy for the treated samples. The experiment was done in three replicates. The labeling efficiency for each sample was checked before mixing into a 1:1 ratio. A labeling efficiency of 98% was reached in all samples. The mixed proteins were then injected into a High Field Qexactive coupled to a nanoLC (LC MS/MS).

MS analysis recorded 281456 spectra. A total of 40288 peptides and 6060 proteins were identified among all the three replicates. 3766 proteins were quantified in at least 2 of the 3 replicates. This quantification approach will be elaborated in the discussion section. Among these proteins, 119 were significantly up-regulated and 548 proteins were significantly down-regulated. Figures 15 and 16 show the top 25 up-regulated and down-regulated proteins, respectively. Tables 3 and 4 in Appendix A list the protein names of the top 25 up-regulated and down-regulated proteins, respectively.

Based on gene ontology (GO) annotations, the top 20 significantly enriched biological processes reflecting the significantly up-regulated proteins are depicted in Figure 17. G-protein coupled receptor signaling pathway enrichment indicates that exendin-4 is binding to its G-protein coupled receptor and inducing a signaling cascade. These processes will be elaborated further in the discussion section. The top 20 significantly down-regulated pathways are shown in Figure 18.

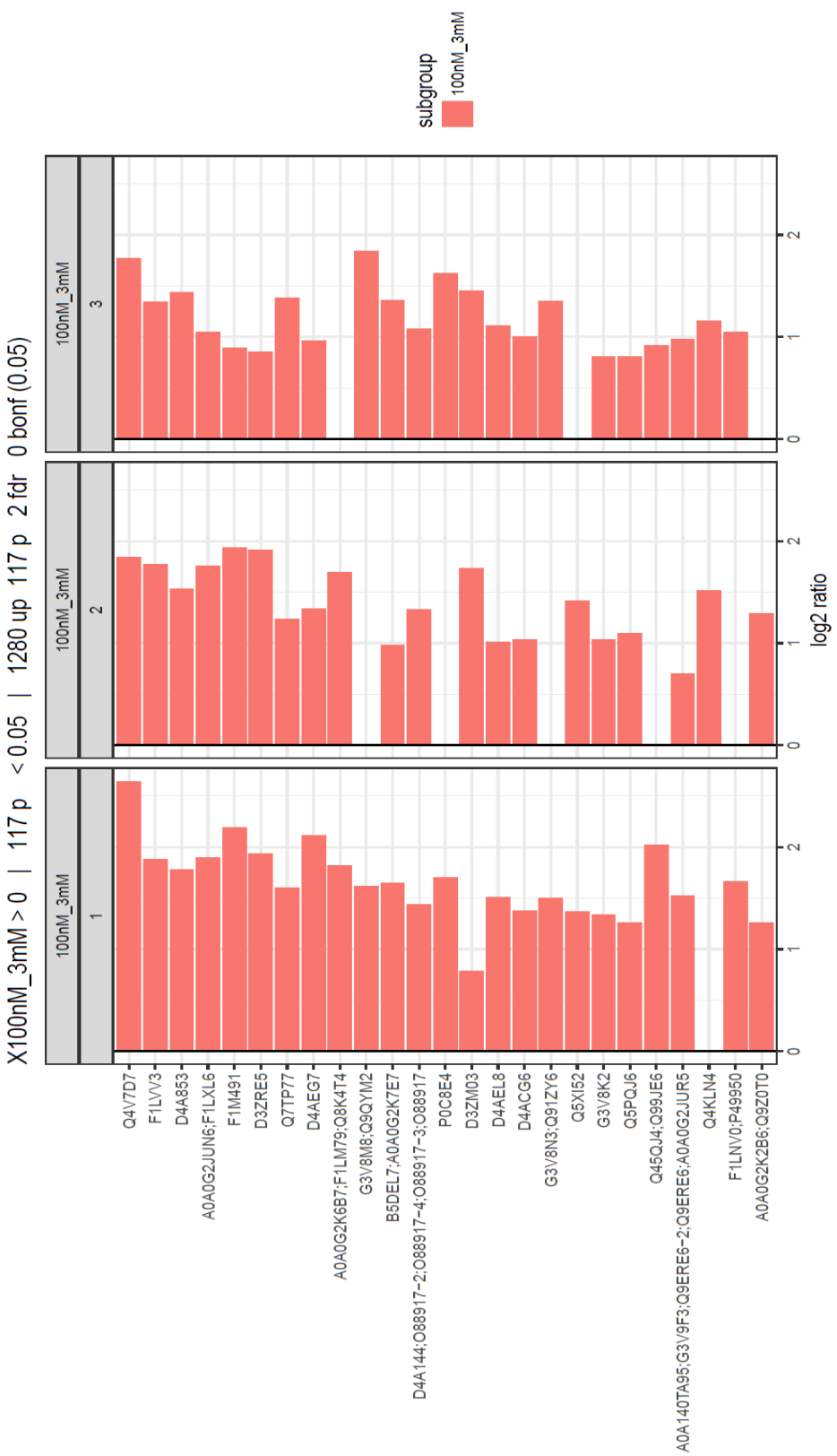

Figure 15: Bar plot representing the top 25 up-regulated proteins in 100nM exendin-4 treated versus control cells. The graph lists the proteins belonging to one protein group and the P value indicating the significance in the change of the expression in at least 2 of the 3 replicates. Among the 1280 up-regulated proteins, 117 were significant.

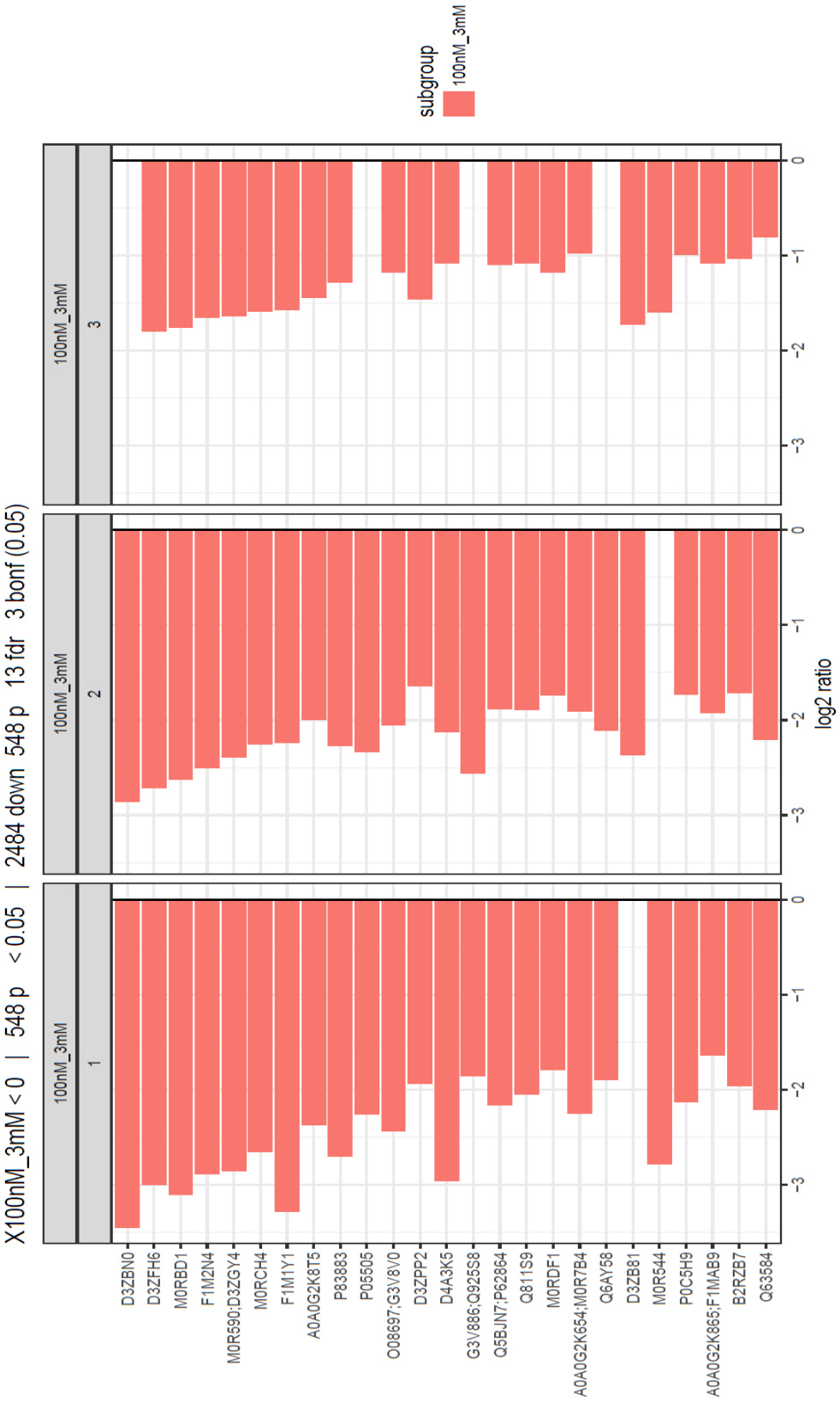

Figure 16: Bar plot representing the top 25 down-regulated proteins in 100nM exendin-4 treated versus control cells. The graph lists the proteins belonging to one protein group and the P value indicating the significance in the change of the expression in at least 2 of the 3 replicates. Among the 2484 down-regulated proteins, 548 were significant.

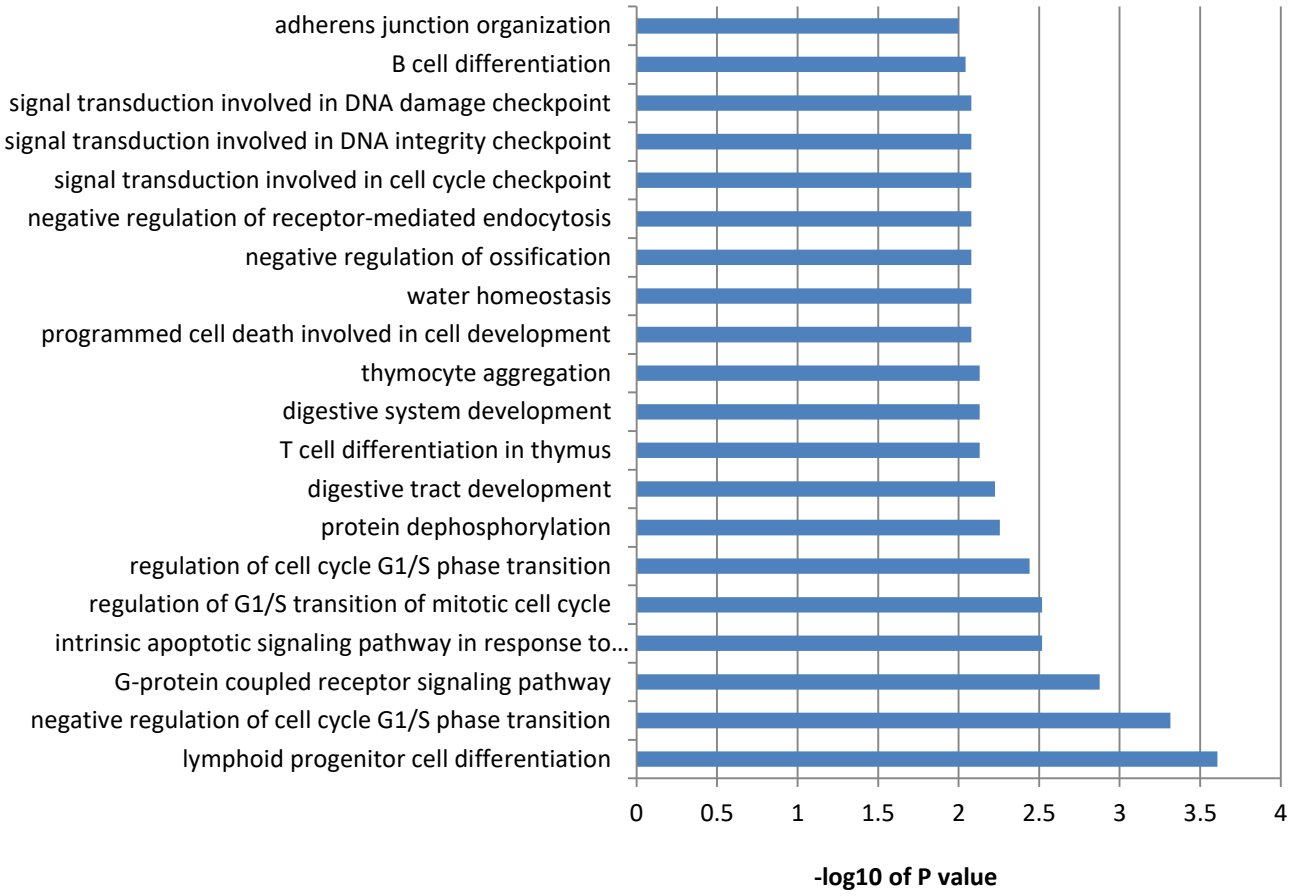

Figure 17: Bar plot of the top 20 significantly enriched biological processes for proteins. Based on gene ontology, the top 20 enriched biological process for proteins up-regulated in 100nM exendin-4 treated versus control cells are depicted in this figure. Values are represented as –log10 of the P value of the significantly enriched biological processes. Each process is assessed by looking at changes in 10 associated proteins.

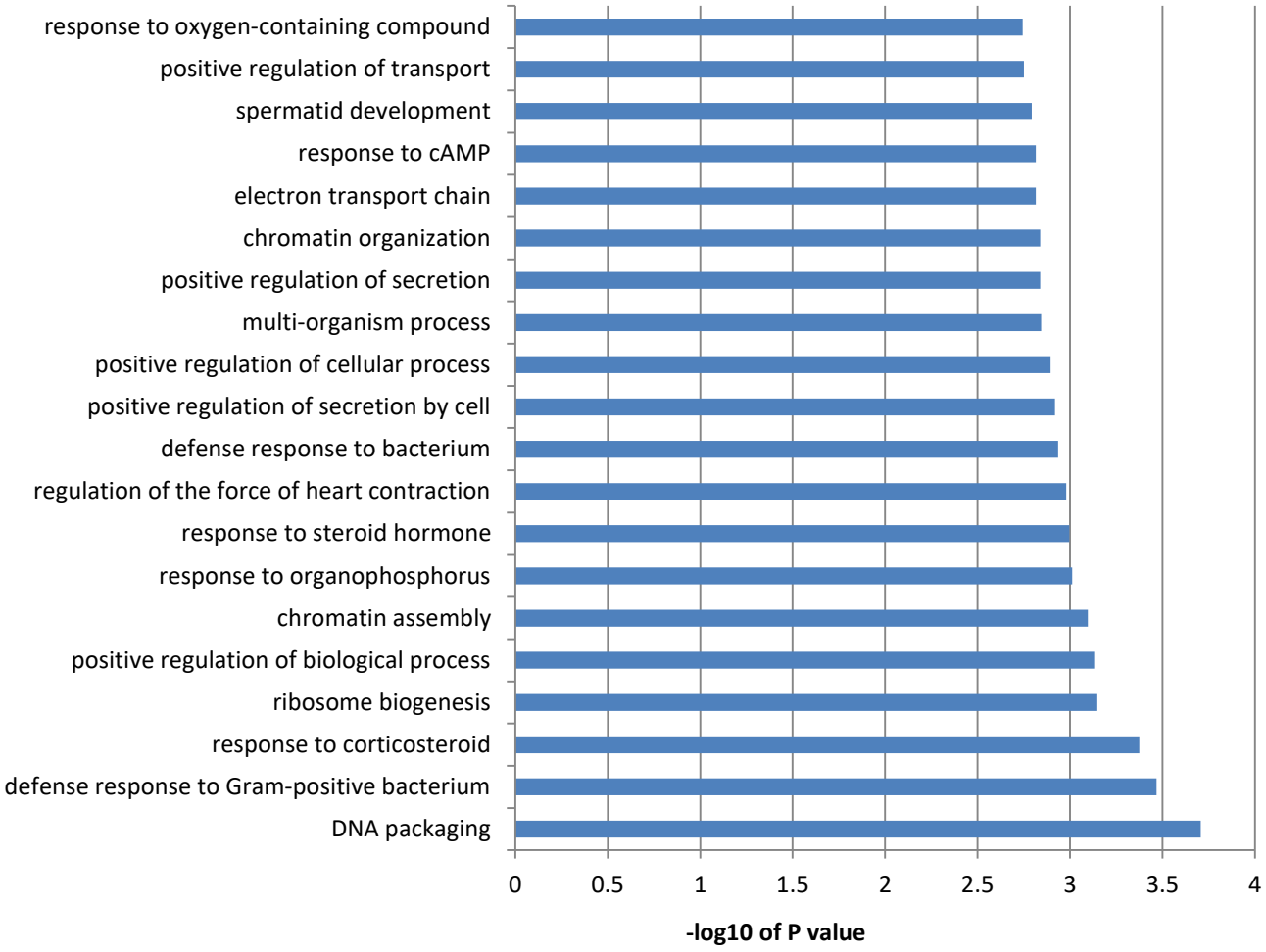

Figure 18: Bar plot of the top 20 significantly depleted biological processes for proteins. Based on gene ontology, the top 20 depleted biological process for proteins down-regulated in 100nM exendin-4 treated versus control cells are depicted in this figure. Values are represented as –log10 of the P value of the significantly depleted biological processes. Each process is assessed by looking at changes in 10 associated proteins.

H. Quantitative phosphoproteome analysis of exendin-4 treated compared with control cells reveals changes in phosphorylation in 400 phosphosites.

For the phosphoproteomic analysis, we subjected the labeled and mixed peptides to enrichment by titanium and IMAC beads using the TiSH protocol by Keller *et al.* (2012). We were able to quantify 1186 phosphoproteins in at least 2 of the 3 replicates. Among these proteins, 2170 phosphosites were quantified. Using our cutoff criteria 60 phosphosites were up-regulated while 340 were down-regulated when comparing exendin-4 treated to the untreated control. These phosphosites are corrected for protein expression and reflect the phosphorylation occupancy, which holds the most significant biological value, according to the following formula:

log2 occupancy = (log2 site.exendin - log2 site.control) - (log2 protein.exendin - log2 protein.control). This approach will be further explained in the discussion section of this thesis. Figures 19 and 20 show the top 20 significantly up-regulated and down-regulated phosphosite occupancies, respectively. Tables 5 and 6 in Appendix B list the protein names of the top 20 up-regulated and down-regulated phosphoproteins, respectively.

Based on gene ontology (GO) annotations, the significantly enriched biological processes reflecting the significantly up-regulated phosphosites occupancies are depicted in Figure 21. Cytokinesis and microtubule bundle formation are among the up-regulated processes indicating that exendin-4 might be playing a role in committing the cells to mitosis. The biological relevance of these processes will be further discussed in the discussion section. The top 15 significantly down-regulated pathways are shown in Figure 22.

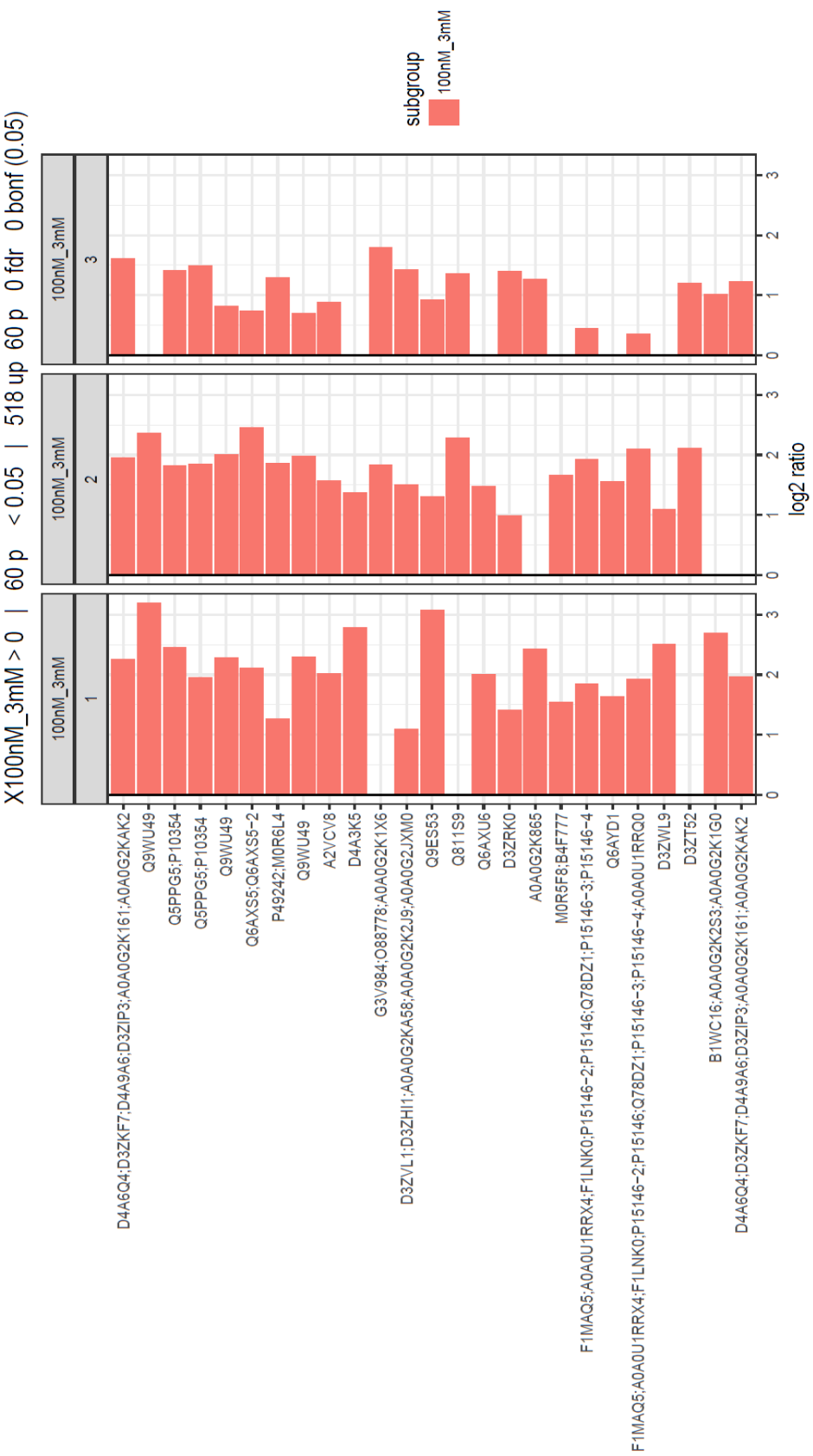

Figure 19: Bar blot representing the top 25 up-regulated phosphosite occupancies in 100nM exendin-4 treated versus control cells. The graph lists the phosphoproteins belonging to one protein group and the P value indicating the significance in the change of the expression in the phosphosite in at least 2 of the 3 replicates. Among the 518 up-regulated sites, 60 were significant.

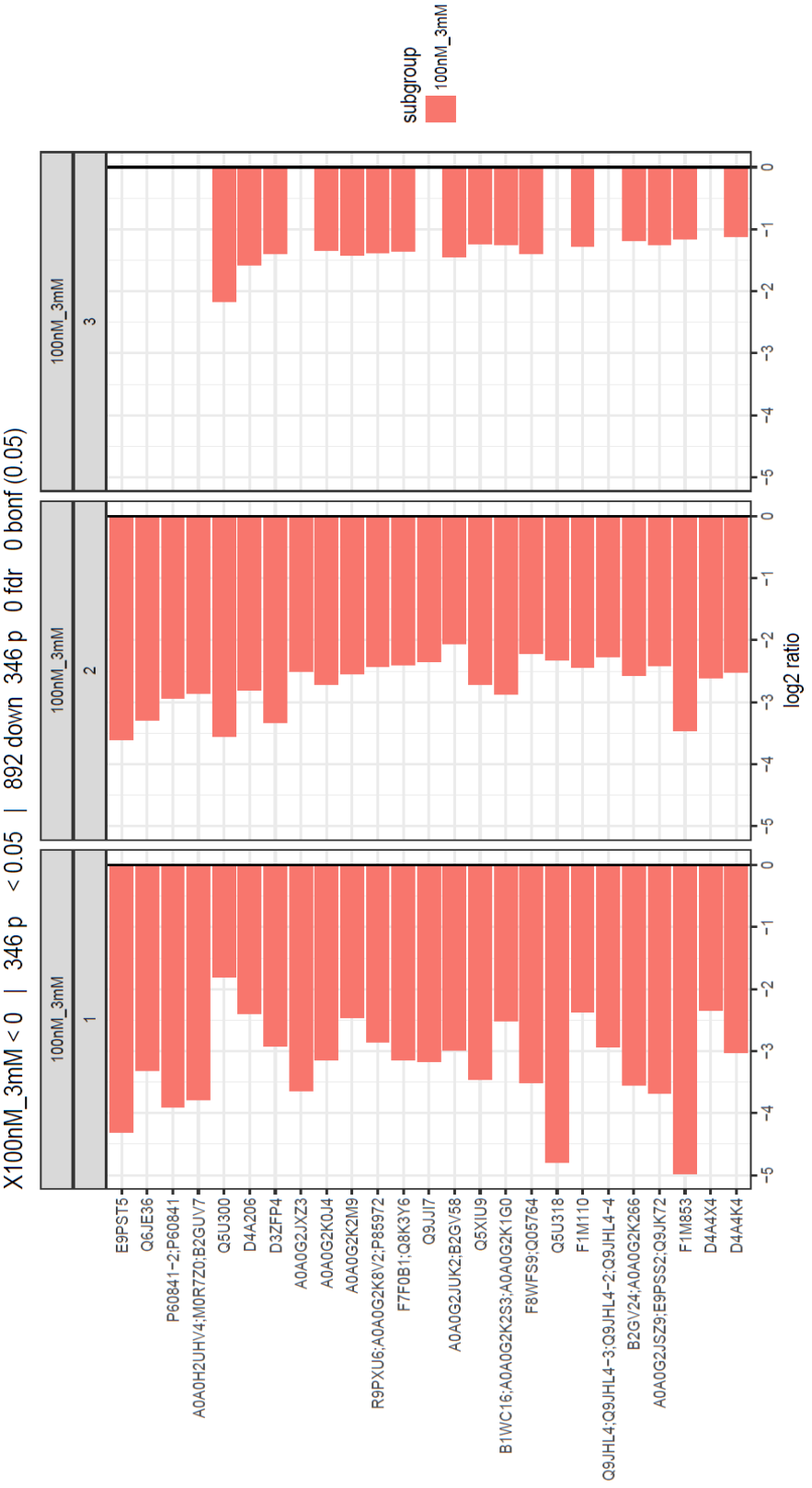

Figure 20: Bar blot representing the top 25 down-regulated phosphosite occupancies in 100nM exendin-4 treated cells. The graph lists the phosphoproteins belonging to one protein group and the P value indicating the significance in the change of the expression in the phosphosite in at least 2 of the 3 replicates. Among the 892 down-regulated sites, 346 were significant.

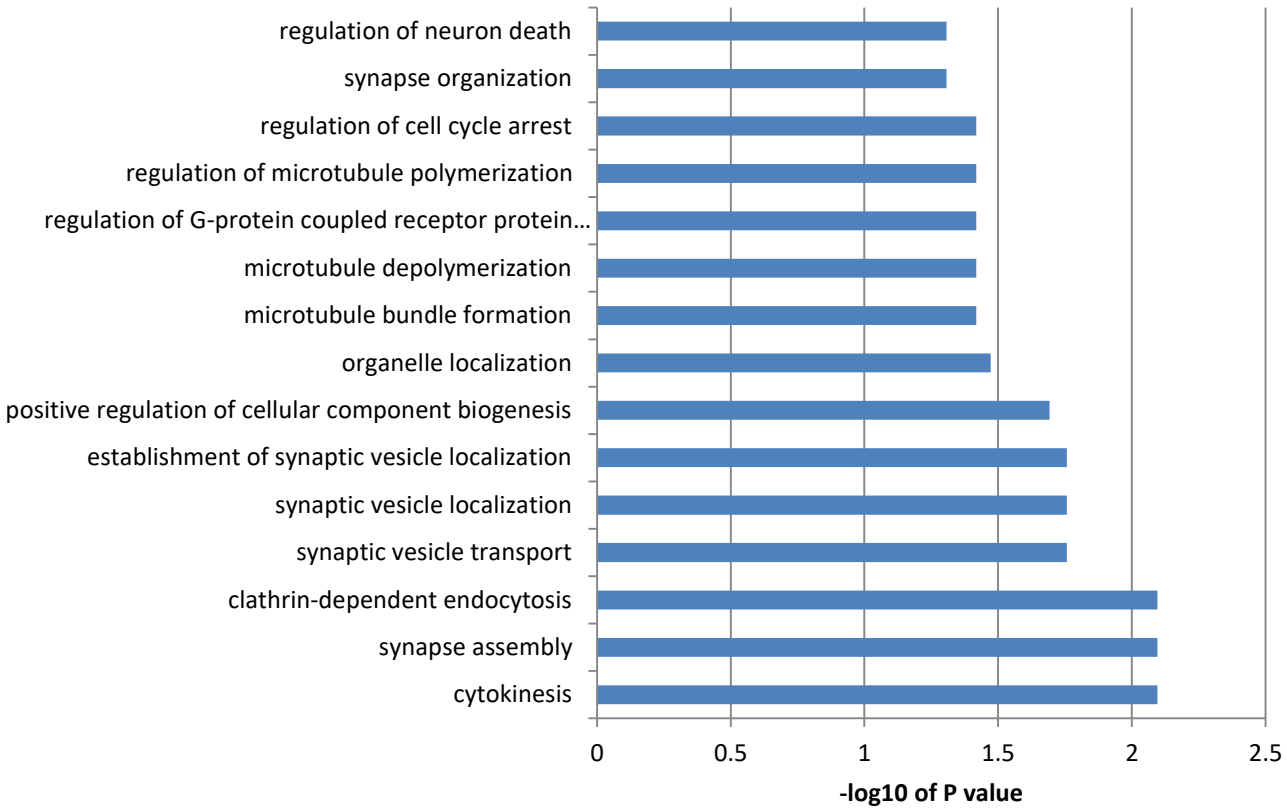

Figure 21: Bar plot of the top 15 significantly enriched biological processes for phosphosites. Based on gene ontology, the top 15 enriched biological process for phosphosite occupancies up-regulated in 100nM exendin-4 treated versus control cells are depicted in this figure. Values are represented as –log10 of the P value of the significantly enriched biological process. Each process is assessed by looking at changes in 10 associated proteins.

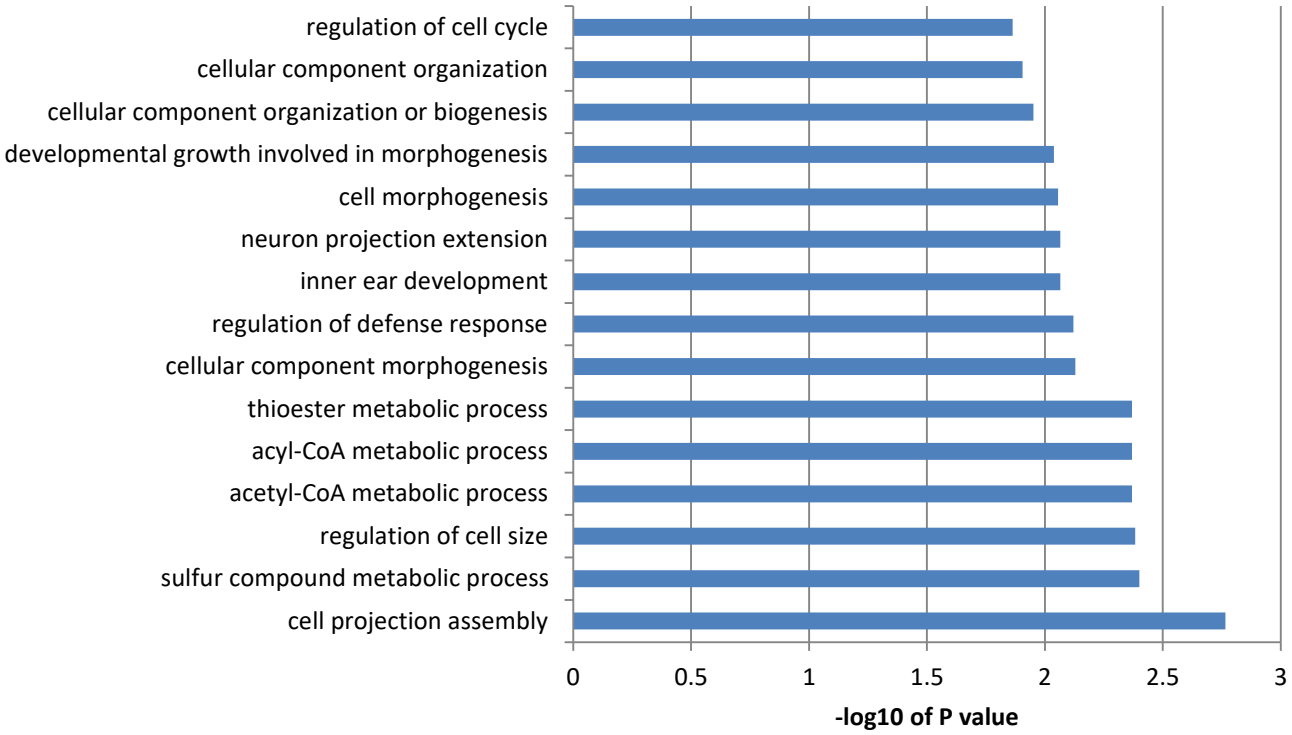

Figure 22: Bar plot of the top 15 significantly depleted biological processes for phosphosites. Based on gene ontology, the top 15 depleted biological process for phosphosite occupancies down-regulated in 100nM exendin-4 treated versus control cells are depicted in this figure. Values are represented as –log10 of the P value of the significantly enriched biological process. Each process is assessed by looking at changes in 10 associated proteins.

Discussion

The study presented here is the first combined proteomic and phosphoproteomic analysis of the effects of treatment with the GLP-1 analogue exendin-4 on a pancreatic beta cell model. Other proteomic studies have been conducted but in the context of highlighting the protective role of GLP-1 and its analogues in stress-induced beta cells (66,75,76), in addition to one transcriptomic analysis of human and rat islets treated with GLP-1 (115). Interest in identifying the proliferative effects underlying exendin-4 treatment stems from the fact that it is a stable analogue to GLP-1 that is already used in the clinic for diabetes treatment as an insulin production enhancer (116). While ins-1 cells derive from a tumor and may not well represent a primary beta cells, it remains a common and well-studied model system especially in terms of cell cycle regulation, survival and apoptosis mechanisms which serves as a first line of study that could be translated to isolated primary islets. A group in France recently established a possibly more medically relevant human pancreatic beta cell model (111), access to which was unfortunately barred to us due to institutional differences with respect to intellectual property.

We started our study by verifying whether the ins-1 cell line represented viable model for the effects of exendin-4 treatment. In the process, the cells responded to glucose, yet insignificantly, and exendin-4 treatment showed an additive effect on insulin secretion with glucose. We were able to recapitulate exendin-4 effects known from the literature such as protein kinase B (PKB) phosphorylation and the stimulation of beta cell proliferation. Based on our observations, we wanted to extend our study and determine how the observed phenotypes translate into changes at the proteomics level. Among the cells tested, we chose the parental ins-1 cell line as it shows lower doubling time compared to its derivatives and has been extensively studied in terms of GLP-1 effects. To establish the assay conditions for the proteomic and phosphoproteomic experiments, we tested the effect of exendin-4 treatment on PKB phosphorylation in complete growth media versus starvation media. In the former no effect of exendin-4 on PKB phosphorylation was detectable, likely due the presence of insulin and other growth factors. Thus, for the preferable starvation condition,

we excluded the possibility that the PKB phosphorylation observed stems from its known starvation-specific activation (114), rather than exendin-4 treatment. Taken collectively, the results led us to conclude that starvation is the method of choice for the cell culturing condition prior to proteomic screening for exendin-4 effects.

The effects of the incretin hormone GLP-1 and its analogues on pancreatic beta cells has long been studied and investigated. These studies were, however, of a targeted nature, focusing on specific pathways or highlighting the protective role of this hormone in stress conditions, as discussed in the introduction section of this thesis. In our study, we aimed at a global proteomic and phosphoproteomic molecular screen for effects of treatment with exendin-4 in comparison to an untreated control. Represented by *ad-hoc* search results in PubMed for *Homo sapiens*, *Mus musculus* and *Rattus norvegicus* (March 2017), that yields 58130964, 575485 and 281425 entries respectively, annotation in *Rattus norvegicus,* is comparatively sparse. The following interpretation of the results of the screen thus heavily relies on homology-based extrapolation.

The fact that we performed the exendin-4 treatment followed by the proteomic screen under starvation conditions added another level of complexity to the analysis. Despite the complications, we expected a proliferative effect of exendin-4 in terms of the alteration of expression of specific proteins. Surprisingly, we observed instead the proteinaceous hallmarks of a protective stress response including inhibited proliferation and apoptosis, with the latter observation being reported previously after GLP-1 treatment (63). In addition to the protective role, exendin-4 was observed to activate a G-protein coupled receptor response and its downstream signaling, implying that the cells responded to exendin-4 treatment even after prolonged starvation.

We started our proteomic analysis by looking at the list of regulated proteins in all three replicates. By analyzing the top 40 proteins with the lowest p value (<0.01), exendin-4 was shown to activate G-protein coupled receptor signaling and to inhibit transcription and proliferation. In addition, exendin-4 up-regulated some proteins involved in the stress response pathway. However, no protective or

anti-apoptotic effects, both of which have been previously reported after exendin-4 treatment, were detected. In addition, the enriched gene ontology biological processes after exendin-4 treatment were generic and did not relate to any of the known functions of exendin-4. Consequently, we decided to be less stringent, and to look at the list of regulated proteins expressed in at least two of the three replicates. The less stringent conditions revealed more protective effects of exendin-4, which were also reflected in the enriched and depleted biological pathways. What follows will be based on the latter approach of quantification for both the proteomic and the phosphoproteomic analysis.

The proteomics screen produced 665 significantly altered proteins, among which we focused on the top 40 with the smallest p values (<0.01) for detailed analysis, integrating information from Uniprot (117) the Rat Genome Database (118) and PubMed. The activation of the G-protein coupled receptor signaling pathway expected from the literature was represented, in our experiment, by the up-regulation of the guanine nucleotide-binding protein subunit gamma (Gng3). G-protein coupled receptors, including GLP-1, are coupled to heterotrimeric G proteins, made of alpha, beta and gamma subunits. Upon activation of the receptor, the alpha subunit dissociates from the complex and binds to the receptor. Both the receptor-bound alpha subunit and the free beta-gamma subunits bind to and activate different downstream effector molecules (119) resulting in ion-channel activation, calcium influx and vesicle formation (120).

In accordance with the vesicle formation endpoint of GPCR signaling and insulin secretion in general, proteins associated with vesicular trafficking and exocytosis including phospholipase C βeta 3, cdk5-p39 fer-1-like family member 6 (Fer1l6), latrophilin and Vma21, vacuolar ATPase assembly integral membrane, were up-regulated by exendin-4 treatment. Phospholipase C beta 3 is a key protein in the signal transduction pathway of G-protein coupled receptors (121). Cdk5-p39 has been shown to induce insulin exocytosis in pancreatic beta cells when overexpressed (122) and Fer1I6 was recently shown to be expressed in several human tissues including the pancreas, and localizes to the trans-

golgi recycling network (123). Latrophilin is alpha-latrotoxin receptor, known as a G-protein coupled receptor which stimulates exocytosis and is expressed in beta cells (124–126).

The above mentioned observations may all be linked to the insulin secretory effects that GLP-1 and its analogues have on beta cells. Contrary to that, we found that Tumor protein D52 (Tpd52), which is known to play a role in vesicular trafficking is down-regulated. While this appears counterintuitive, the also observed increased phosphosite occupancy at serine 186 of the same protein might compensate for reduced abundance. Studies have shown that Ca^{2+} dependent phosphorylation of this protein at ser-136 positively regulates endosome trafficking involved in cytokinesis in proliferating cells (127) and activation of the lysosome-like secretory pathway in pancreatic acinar cells (128). The site at ser-186 has not been described before, but we hypothesize it to have a similar phenotype.

As mentioned earlier, the responses we saw after exendin-4 treatment were mainly protective-stress responses due to the serum starvation condition. This is evident through the up- and down-regulation of proteins with diverse functions. Exendin-4 treatment seems to repress transcription by up-regulating the transcription repressor capicua transcriptional repressor (Cic) and down-regulating the transcription factor BTF3 and Lysine demethylase 3B (Kdm3b). Cic's inactivation has been shown to drive cancer metastasis (129,130), which is contrary to what we see after exendin-4 treatment. The protein's transcription repressor function is emphasized in our data by a down-regulation of phosphorylation at ser-173. The down-regulation of phosphorylation at Cic serine-173, as we also observed following exendin-4 treatment, activates Cic by preventing its binding to the 14-3-3 family of proteins, further suggesting that exendin-4 drives its transcription repressor function (131). BTF3, also known as nascent polypeptide-associated complex b (NACB) is comprised of two subunits where subunit A holds the transcriptional function by binding to RNA polymerase and was shown to be up-regulated in human pancreatic ductal adenocarcinoma (132), the role of which explains its down-regulation by exendin-4 as an inhibitor of proliferation in the starvation condition employed in our experiment. Kdm3b is a methylase that removes methyl groups from lysine residues on histones and

consequently promotes transcription (133). Such an effect would be reversed in our case since the methylase enzyme is down-regulated.

Hallmarks of repressed proliferation in our study are the down-regulation of nucleostemin, mesencephalic astrocyte-derived neurotrophic factor (Manf), ATP-dependent zinc metalloprotease (YME1L1), large subunit ribosomal protein L36a (Rpl36a), ADP-ribosylation factor-like protein 2 (Arl2), ADP-ribosylation factor-like GTPase 8A (Arl8a) and histone linker proteins H1.1, H1.2 and H1.5. Nucleostemin is a GTPase protein known to play a role in cell cycle progression since depletion of the protein causes defects in the cell cycle phase transition (134). We found that nucleostemin phosphorylation at thr-496 was up-regulated. This site has not been reported previously which renders it a potential candidate for future studies involving exendin-4 treatment. Manf is a protein released due to ER stress and was shown recently to be crucial for the growth of pancreatic beta cells in mice and human (135). YME1L1 is a mitochondrial inner membrane protease crucial for maintaining mitochondrial proteostasis and proliferation induction (136). Exendin-4 seems to be shutting down mitochondrial processes, since in addition to YME1L1, solute carrier family 25 member 31 (Slc25a31), belonging to a family of mitochondrial proteins that transports solutes across the mitochondria by exchanging ATP for ADP (137), was down-regulated. We hypothesize that this could be a mechanism by which exendin-4 is putting the starved cell in an energy saving mode. As the name indicates, Rpl36a, is a ribosomal protein subunit required for protein translation and its overexpression in hepatocellular carcinoma contributes to tumor growth (138), a regulation that supports our conclusion that proliferation is inhibited by exendin-4 in starved cells. Arl2 and Arl8a belong to a family of GTPases and have been recently characterized as positive regulators of microtubule-dependent processes and chromosomal segregation (139). Having these two proteins significantly down-regulated supports a growth inhibition in the starved state by exendin-4. The H1 histone linker family of proteins, H1.1, 1.2 and 1.5, are components of chromatin which determine its higher structural organization and are mainly up-regulated during S phase (140). In spite of the fact that H1.1 is down-regulated in our experiments, phosphorylation at ser-106 is up-regulated. Histone H1 phosphorylation (no sites specified) is known to peak during interphase and mitosis, with H1.1

phosphorylation mainly occurring at interphase (141). This observation may hint at a role for exendin-4 treatment in establishing a prone cell cycle initiation state which fails to progress due to the starvation condition of the cells.

The regulation of the previously discussed proteins and their functions was also observed in the significantly regulated list of proteins quantified in all three replicates. We were curious to know what other phenotypes could be detected if we opened up the search filters and used less stringent conditions. Despite the anti-proliferative effects observed after exendin-4 treatment, some of the significantly altered proteins point to a protective role that exendin-4 is playing as a response to the stress induced by serum starvation, where inhibition of proliferation could also be a defense mechanism constraining the cells from dividing in stress conditions. Centrosomal protein 131 is a centriolar satellite protein (142) that we observed to be up-regulated after exendin-4 treatment. UV stress mediated P38/MAPK-dependent phosphorylation of Cep131 at ser-47 and 78 leads to cytoplasmic sequestration (143) away from the centrioles to stop centrosome duplication. Interestingly, phosphorylation of this protein at ser-47 was down-regulated in our case suggesting that exendin-4 is partially reversing the effects of stress induction responses by p38 and maintaining its localization at the centrioles in support of a prone state with respect to initiation of mitosis. RAN binding protein 9 (Ranbp9, also known as RanBPM) is a nucleocytoplasmic protein with diverse biological functions (144) which is known to induce stress responses to DNA damage (145,146). This is in accordance with what we found since Ranbp9 was up-regulated by exendin-4 treatment. Chromodomain helicase DNA-binding protein 1-like (Chd1l) was identified by Ahel *et al.* (147) as a chromatin remodeling enzyme which recruits poly(ADP-ribose) (PAR) to DNA damage sites. In agreement with that, we saw that PAR glycohydrolase (PARG) which removes the PAR group polymerized by PAR polymerase (PARP) at DNA breaks was also up-regulated. The breakdown of the PAR group by PARG generates adenosine triphosphate (ATP) required for DNA repair (148). Gle1 is a regulator of translation playing a role in initiation and termination (149) and is found to be up-regulated after exendin-4 treatment. Human Gle1 has recently been shown to involve in stress granule formation (150), which supports our hypothesis of stress response induction by exendin-4.

During cellular stress, such as nutrient deprivation, translation is silenced and pre-mRNAs are assembled into stress granules (151). Stress granules are heterogeneous populations containing splicing factors, repressors and regulators of mRNA stability (152). This might explain the significant up-regulation of the pre-mRNA-splicing factor RBM22, whose function is still under investigation but is part of the spliceosomal complex (153), after exendin-4 treatment.

Supporting the above facts and our argument that exendin-4 is playing a protective role, we saw that anti-apoptotic proteins such as bcl-2 and Mitogen-activated protein kinase kinase kinase 7 (Map3k7 or TAk1) were up-regulated. Bcl-2, B Cell Lymphoma/Leukaemia 2, is a well-known anti-apoptotic or prosurvival protein discovered in 1988 (154). After stress induction, Bcl-2 inhibits the binding of the pro-apoptotic Bcl-2 family proteins BAX and BAK thus protecting the mitochondrial outer membrane from permeabilization (155). TAK1 was first identified in 1995 by Yamaguchi K *et al.* (156) as a regulator of the TGF-β signaling pathway. TAK1 is activated in response to a stress stimulus such as nutrient deprivation resulting in activation of NF-κB which in turn induces the expression of anti-apoptotic proteins (157). However, TAK1 phosphorylation was down-regulated at ser-417 and 439 after exendin-4 treatment. Phosphorylation at these sites has been previously reported (100) but nothing is known about regulation of protein function through it. We did, however, not see phosphorylation at thr-187 in the activation loop, which is essential for the activation of TAK1 kinase activity when cells are stimulated with TNF alpha (158). This may suggest that exendin-4 activates effectors upstream of TAK1 but is not able to induce TAK1 phosphorylation. The 40S ribosomal protein S30 protein, also known as Fau was down-regulated. Fau has been shown to play a role in apoptosis regulation by acting as a tumor suppressor (159,160), the effect of which is reversed due to the down-regulation of the protein, suggesting a role for exendin-4 in maintaining cell survival. The down-regulation of B-cell receptor-associated protein 31, Bcap31, supports our argument for the protective role played by exendin-4. This is an ER protein known to be involved in ER-mediated apoptotic responses by acting as a substrate for caspase 8 cleavage which in turn triggers the release of other caspases and promotes apoptosis (161). As mentioned above, we observed a down-regulation of the histone linker protein H1.2 (Hist1h1c). This might have dual opposing consequences

on the cells after exendin-4 treatment. Histone linker proteins are required for higher structure chromatin remodeling (162) essential for cell division, but it appears that H1.2 has an extended role as a pro-apoptotic protein inducing mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release (163). Taken together, we conclude that exendin-4 is inhibiting cell division due to the stress conditions yet maintaining cell survival by inhibiting apoptosis.

The above interpretations are reflected by the enriched and depleted Gene Ontology Biological Processes (GOBP) observed after exendin-4 treatment. Among the enriched pathways are the G-protein coupled receptor signaling, intrinsic apoptotic signaling in response to DNA damage and negative regulation of cell cycle G1/S phase transition explained by stabilization of the cytoskeleton and phosphorylation of histone H1.1 which might be pushing the cells to interphase but inhibiting the transition to the other mitotic stages due to DNA damage. On the contrary, DNA packaging, ribosome biogenesis and chromatin assembly processes were depleted, reflecting the down-regulation in transcription factors and histone binding proteins. Our data agrees with published reports on a protective role of exendin-4 and GLP-1 since the latter counteracts cell death caused by cytokines in human islets (75). In addition, exendin-4 protected rat ins-1 cell lines from glucolipotoxicity-induced ER stress through down-regulation of SREBP1c (65). Liraglutide improved rat beta cell survival via the AMPK/mTOR/P70S6K signaling pathway and protected the cells against glucolipotoxicity by activating mTOR (48).

As shown in the results section, we were able to quantify 406 phosphosite occupancies, present in at least 2 of the 3 replicates. Phosphosite occupancies reflect the true relative level of increased or decreased phosphorylation corrected for the level of the protein. To illustrate, plain phosphosite quantitation may yield signals due to changing protein abundance. Phosphosite occupancies, as used here, reflect changing phosphorylation levels independent of protein abundance. This data, in our opinion, harbors most relevant biological information. We focused on a subset (p value <0.01) of the top significantly regulated phosphoproteins, and their potential interactions. Data published to date

holds little information on these proteins, especially in terms of regulation by phosphorylation. Therefore, most of what we report here is novel and may warrant further investigation.

In addition to the phosphorylation regulation already discussed in the context of protein abundance changes above, we saw significant up-regulation of phospho-occupancies on ser-32, 41 and 52 of the calcium-regulated heat-stable protein 1 (Carhsp1). This protein is regulated by calcium and localizes to the apical side of secretory epithelial cells (164). Phosphorylation and regulation of these sites by signaling from the growth factors IGF-1, PKBα and EGF have been reported previously in rat liver and pancreatic acinar cells (165,166). Interestingly, the protein itself was found to be down-regulated by exendin-4 treatment. This apparent discrepancy may indicate competing effects by the starvation treatment employed by exendin-4 exposure or may support the two-pronged approach of shutting down a protein activity, by reducing abundance and inducing inhibitory phosphorylation.
Analogously, plasminogen activator inhibitor 1 RNA-binding protein (Serbp1) was also down-regulated while phosphosite occupancy at ser-393 and 378 was up-regulated. Serbp1 binds to and stabilizes plasminogen activator inhibitor-1 (PAI-1), found overexpressed in solid tumors like ovarian and breast cancer (167,168). SERBP1 is further crucial for DNA repair during S phase and binds to and stabilizes CtBP-interacting protein (CtIP) which is in turn important for repairing double strand breaks during homologous recombination (169). The observed phosphosite occupancy up-regulation at ser393 and 378 has not been previously reported in rat and we hypothesize, based on what was discussed earlier, that exendin-4 mediated the induction of phosphorylation to counteract down-regulation of the protein expression and thus correct for a potential reduction of DNA repair capacity.

A similar inverse correlation between protein abundance and phosphorylation at ser-521 and/or 522 was observed for Daxx. The two up-regulated phosphosites are novel to the best of our knowledge and the information compiled in the UniProt Database. Daxx is a protein that acquires several functions depending on its cellular localization, including transcriptional corepression (170). Given the protein's expression phenotype observed after exedin-4 treatment and its functional annotation, phosphorylation up-regulation at the specified sites may act as an activator of the protein further

supporting the transcriptional repression profile after exendin-4 treatment. Serbp1 is known to interact with Daxx (169), which might explain the down-regulation in protein abundance for both, while the phosphosites up-regulation remains an interesting area that is worth exploring.

Two of the most important enriched Biological Processes based on Gene Ontology from up-regulated phosphosite occupancies are “cytokinesis” and “microtubule bundle formation and depolymerization”. Cytokinesis is centrally represented in our experiment by the up-regulation of phosphorylation on ser-16 in stathmin 1 protein and ser-313 in Plakophilin-4 (pkp4) also known as p0071. Phosphorylation of stmn1 at ser-16 is crucial for its role in microtubule depolymerization by increasing the amount of free cytoplasmic tubulin available for microtubule assembly (171). Plakophilin-4 (pkp4) plays a role as a positive regulator of Rho activity during cytokinesis (172). Phosphorylation of this protein at ser-313 has been previously reported in mice (Uniprot) without any indication of function. Enrichment of microtubule bundle formation-related terms was driven by phosphorylation up-regulation of microtubule-associated protein 1B (Map1b), microtubule-associated protein 2 (Map2) and stathmin 1. Phosphorylation of ser-1776, 1779 and 1781 in Map1b has been previously reported (104,173). Phosphorylation at the three serine sites was up-regulated after exendin-4 treatment, as well as phosphorylation at ser-1816 and 1611 in Map2, which is known to bind to and stabilize microtubules (174). Map1b is known to be one of the major cytoskeletal proteins in neurons and is crucial for stabilizing the structure of microtubules (173). An interesting observation in this context is that all of the above listed proteins were down-regulated while their reported phosphorylation sites are up-regulated bringing us again to the conclusion that exendin-4 might be counteracting the effect of starvation by activating proteins that prepare the cells for mitosis or may be inducing phosphorylation at the mentioned sites to inhibit the protein activity. On the other hand, among the highly significantly up-regulated proteins (with no phosphorylation sites identified) is myosin phosphatase Rho-interacting protein (Mprip). G-protein coupled receptor signaling inhibits myosin phosphatase, thus keeping myosin in the phosphorylated state and causing smooth muscle cells contraction through the activation of RhoA/Rho kinase (175). This phosphorylation stabilization of myosin is crucial for its binding to actin filaments and consequently involving in centrosome

separation and spindle assembly (176). Owing to the above discussed observation of the protective, anti-proliferative role of exendin-4, another possible hypothesis could be that the changes in cytoskeletal regulating proteins account mainly for vesicular trafficking induced by GLP-1 receptor activation and do not relate to any mitotic activity, especially given the fact that microtubule and chromosome segregation proteins, Arl2 and Arl8a, are down-regulated.

Some of the proteins with significantly regulated profiles are either not well studied or characterized or their functions hold no apparent relevance to the exendin-4/GLP-1 complex. For instance, Phb-ps1 was among the down-regulated proteins. It remains uncharacterized in rats (Uniprot) but shares 93% sequence homology with mouse, but not rat, prohibitin. The mouse protein has been recently shown to be crucial for beta cell survival in mice (177). The characterization of the Phb-ps1 protein and its function in rat islets may provide another hint towards the understanding of growth mechanisms in these cells. Similarly, small ribonucleoparticle associated protein (Snrpn) is also an uncharacterized protein that shares 90% sequence homology with human 60S ribosomal protein L24 (RPL24) and was down-regulated after exendin-4. The human protein has been shown to be overexpressed in breast cancer tissues compared to normal controls (178). If the uncharacterized protein shares the same function with RPL24, then its down-regulation is expected in the context of an inhibition of growth by exendin-4. In addition, four of the significantly down-regulated proteins, namely M0RCH4, F1M1Y1, F1M2N4 and M0RBD1 are not yet characterized. Aside from MORCH4 and F1M2N4, which show 60% and 88% sequence homology with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively, sequence homology analysis using the Uniprot database produces no further hints as to their function. The homologies found are in agreement with the observation that GAPDH was down-regulated after exendin-4 treatment, probably due to the low glucose level in which the cells where cultured. Mitochondrial ribosomal protein L49 (Mrpl49) was up-regulated after exendin-4 treatment. This protein is found in the large ribosomal subunit of the mitochondria (179), but no reports characterizing its function have been published yet, based on a PubMed search done on 22-4-16. TBCd13, is a TBC1 domain family which is also not well studied but worth investigating in future implications of exendin-4 treatment. Among the proteins that show no apparent relevance in terms of

function are lysosomal alpha glucosidase (Gaa), which is essential for degradation of glycogen to glucose in lysosomes (Uniprot) and subunit 3 of cytochrome oxidase (Mtco3) is part of the oxidase complex which is the last enzyme in the mitochondrial electron transport chain (Uniprot), where down-regulated, while Sec61 gamma which is a component of the mammalian translocon and is required for the translocation of peptides to the ER lumen (180), was up-regulated. These proteins may be prime candidates for novel affectors by exendin-4 and thus likely GLP-1. A connection to exendin-4 treatment is to be validated by further studies.

Taken collectively, we present a proteomic and phosphoproteomic screen for the effects of exendin-4 treatment in the rat pancreatic insulin producing beta cell line ins-1, which is the first of its kind. While the agent is known to have a proliferative effect, our results seem to indicate a more protective effect of exendin-4 due to the stress induced by the starvation condition employed. This observation is in support of other reports that stress is mitigated by exendin-4 treatment and other analogues of GLP-1, as mentioned in the introduction section of this thesis. The apparent discrepancy between the enhanced proliferation observed using the Brdu incorporation assay and an apoptosis protective role observed after the proteomic screen could be attributed to the enhanced Brdu incorporation seen after DNA repair (181,182). Despite the limitations that our study holds with respect to insignificant glucose responsiveness and the harsh starvation conditions, it serves as a platform for developing further studies. Firstly, it would be helpful to repeat the proteomic profiling under less harsh conditions, using, for instance, a shorter serum starvation period or complete growth media. Secondly, performing a proteomic screen of starved cells in comparison to normally growing cells provides a background of proteins altered during starvation and consequently allows observation of whether exendin-4 is correcting the starvation-induced alterations. Last but not least, knockdown of the top regulated proteins is crucial to validate their role.

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Appendix A

Table 3: list of Up-regulated proteins

Gene name	Protein name
Rbm22	Pre-mRNA-splicing factor RBM22
Ranbp9	RAN-binding protein 9
Cic	capicua transcriptional repressor
Fer1l6	fer-1-like family member 6
Cdk5r2-p39	Cyclin-dependent kinase 5-p39
LOC499746	STPG3, Sperm-tail PG-rich repeat-containing protein 3
Mrpl49	mitochondrial ribosomal protein L49
Tbc1d13	TBC1 domain family, member 13
Filip1	Filamin-A-interacting protein 1
Parg	Poly(ADP-ribose) glycohydrolase
LOC100363239	Vacuolar ATPase assembly integral membrane protein Vma21
Adgrl1;Lphn1	Latrophilin-1
Map3k7	Mitogen-activated protein kinase kinase kinase 7
Cep131	Centrosomal protein 131
Chd1l	Chromodomain helicase DNA-binding protein 1-like
Psmc3ip	Homologous-pairing protein 2 homolog
Vezt	Vezatin
Gng3	Guanine nucleotide-binding protein subunit gamma
Pycrl	Pyrroline-5-carboxylate reductase 3
Plcb3	Phosphoinositide phospholipase C
Mprip	Myosin phosphatase Rho-interacting protein
Gle1	Nucleoporin GLE1
Bcl2	Apoptosis regulator Bcl-2

Table 4: list of down-regulated proteins

Gene name	Protein name
Hist1h1b	Histone H1.5
Phb-ps1	93% sequence homology with mouse prohibitin
RGD1560821	Similar to small nuclear ribonucleoparticle-associated protein
F1M2N4	60 % homology to GAPDH
LOC685186;Gapdh-ps2	Glyceraldehyde-3-phosphate dehydrogenase
M0RCH4	homology to GAPDH
F1M1Y1	homology to GAPDH
BTF3	Transcription factor BTF3
Rpl36a	60S ribosomal protein L36a
Mtco3	Cytochrome c oxidase subunit 3
Arl2	ADP-ribosylation factor-like protein 2
Arl8a	ADP-ribosylation factor-like GTPase 8A
Hist1h1a	Histone H1.1
Yme1l1	ATP-dependent zinc metalloprotease YME1L1
LOC100360647;Fau	40S ribosomal protein S30
Gnl3	Guanine nucleotide-binding protein-like 3, nucleostemin
Kdm3b	Lysine demethylase 3B
LOC684828	Histone H1.2
Bcap31	B-cell receptor-associated protein 31
Slc25a31	Solute carrier family 25 member 31
Gaa	lysosomal alpha glucosidase
Manf	Mesencephalic astrocyte-derived neurotrophic factor
Tpd52	Tumor protein D52
Snrpd1	Small nuclear ribonucleoprotein D1

Appendix B

Table 5: list of up-regulated phosphoproteins

Gene name	Protein name
Epb4.1	Erythrocyte membrane protein band 4.1
Carhsp1	Calcium-regulated heat stable protein 1
Chga	Chromogranin-A
Serbp1	Plasminogen activator inhibitor 1 RNA-binding protein
Rps3a;LOC100365839	40S ribosomal protein S3a
Mlx	Carbohydrate-responsive element-binding protein
Hist1h1a	Histone H1.1
Bsn	Protein bassoon
Ctage5	cTAGE family member 5
Ufd1l	Ubiquitin recognition factor in ER-associated degradation protein 1
Gnl3	Guanine nucleotide-binding protein-like 3
Hn1	Jupiter microtubule associated homolog 1
Fam83h	Family with sequence similarity 83, member H
A0A0G2K865	Tumor protein D52
Hmgn5	High mobility group nucleosome-binding domain-containing protein 5
Map2	Microtubule-associated protein 2
Mblac1	Metallo-beta-lactamase domain-containing protein 1
Dido1	Death inducer-obliterator 1
Pbrm1	Polybromo 1
Bclaf1	BCL2-associated transcription factor 1

Table 6: list of down-regulated phosphoproteins

Acin1	Protein kinase B
Ndrg1	Protein NDRG1
Ensa	Alpha-endosulfine
Eif5b	Eukaryotic translation initiation factor 5B
Uba1	Ubiquitin-like modifier-activating enzyme 1
Tcof1	Treacle ribosome biogenesis factor 1
Mcm3	DNA helicase
Atrx	Transcriptional regulator ATRX
Nipbl	NIPBL, cohesin loading factor
Srrm2	Serine/arginine repetitive matrix 2
Vcl	Vinculin
Zc3hav1	Zinc finger CCCH-type antiviral protein 1
St14	Suppressor of tumorigenicity 14 protein homolog
Lsm14a	LSM14A mRNA-processing body assembly factor
Pgrmc2	Membrane-associated progesterone receptor component 2
Bclaf1	BCL2-associated transcription factor 1
Add2	Beta-adducin
Pea15	Astrocytic phosphoprotein PEA-15
Nmt2	Glycylpeptide N-tetradecanoyltransferase
Dbnl	Drebrin-like protein
Ufl1	E3 UFM1-protein ligase 1
Ccs	Copper chaperone for superoxide dismutase
Rrbp1	Ribosome-binding protein 1
Cgn	Cingulin
Vps13c	Uncharacterized protein