The Identification of Novel Proteins that Interact with the GLP-1 Receptor and Restrain its Activity

By

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Abstract

G-protein coupled receptors (GPCRs) have been shown to interact with an array of accessory proteins that modulate their function. I hypothesize that the GLP-1R, a B-class GPCR, similarly has interacting proteins that regulate its signaling. An unliganded human GLP-1R was screened using a membrane-based split ubiquitin yeast two-hybrid (MYTH) assay and a human fetal brain cDNA prey library to reveal 38 novel interactor proteins. These interactions were confirmed by co-immunoprecipitation and immunofluorescence. When co-expressed with the GLP-1R in cell lines, 15 interactors significantly attenuated GLP-1-induced cAMP accumulation. Interestingly, SiRNA-mediated knock down of three selected novel interactors, SLC15A4, APLP1 and AP2M1, significantly enhanced GLP-1-stimulated insulin secretion from the MIN6 beta cells. In conclusion, this present work generated a novel GLP-1R-protein interactome, identifying several interactors that suppress GLP-1R signaling; and the inhibition of these interactors may serve as a novel strategy to enhance GLP-1R activity.
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In the early 1900s, it was proposed that the gut releases factors in response to nutrient ingestion, which stimulate the pancreas to secrete a substance capable of reducing blood glucose\(^1\); this pancreatic substance was later identified as insulin\(^2\). Insulin is a crucial hormone that regulates glucose homeostasis. When blood glucose levels rise, insulin is produced and secreted from the pancreatic beta cells. It acts to lower blood glucose levels by increasing glucose uptake by the liver, muscle and adipose tissue and decreasing hepatic gluconeogenesis. The gut factors, on the other hand, are now known as incretins\(^3,4\). Incretins are a group of hormones released from the intestinal mucosa following food intake. They act to augment glucose-stimulated insulin secretion (GSIS) from the pancreatic beta cells, and thus aid in maintaining blood glucose levels. This nutrient-mediated gut response, or “incretin effect”, was confirmed with the development of radioimmunoassays, which demonstrated that oral glucose ingestion induced a more robust increase in plasma insulin levels as compared to intravenous administration of the same amount of glucose\(^5,6\). In fact, the incretin effect accounts for at least 50% of the total insulin secreted after nutrient administration.

Two peptide hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) fulfill the definition of an incretin in humans\(^7\). GIP was discovered in the crude extract of porcine small intestine\(^8\). It is mainly expressed in the stomach and intestinal K-cells, where it is processed and released into the circulation in
response to nutrient ingestion. GLP-1, on the other hand, is encoded by the proglucagon gene\textsuperscript{9}. It is primarily synthesized and secreted by the L-cells of distal ileum and colon. GIP and GLP-1 have been shown to potentiate GSIS in an additive manner, and together they account for the full incretin effect\textsuperscript{7}. The importance of GLP-1 in maintaining glucose homeostasis was demonstrated by a disruption of the murine GLP-1 receptor, which resulted in mild fasting hyperglycemia and impaired glucose tolerance associated with defective insulin secretion following both oral and intraperitoneal glucose challenge\textsuperscript{7}.

\subsection*{1.1.2 Proglucagon Expression and Regulation}

The proglucagon gene comprises 6 exons and 5 introns\textsuperscript{9} (Fig. 1). It encodes a single 180 amino acid precursor protein, which can be processed into several products including glicentin-related polypeptide (GRPP) contained in exon 2, glucagon and oxyntomodulin (OXM) in exon 3, and GLP-1 in exon4 and GLP-2 in exon 5. Proglucagon mRNA is expressed in three types of cells in humans: alpha cells of the pancreatic islet, L-cells of the intestine and neurons located in the caudal brainstem and the hypothalamus\textsuperscript{10,11}. At least 6 homeodomain proteins have been identified to interact with the proglucagon promoter and regulate its activation\textsuperscript{13}. While deletion of some of these transcription factors (Cdx-2, Brn-4 and Nkx6.2) showed no apparent effect on proglucagon production in vivo, suggesting functional redundancy and compensation, expression of the others (Pax-6 and Pdx-1) are proved to be essential for the proper development of proglucagon-producing cells\textsuperscript{13}. Mice with a dominant-negative Pax-6 mutation, for instance, exhibits disrupted islet development and lacked enteroendocrine cells\textsuperscript{14}. Tissue-specificity has also been demonstrated at the level of transcriptional regulation of the proglucagon gene. For example, Wnt signaling-mediated activation of beta-catenin stimulates proglucagon
expression in the intestine but not in the islet cells\textsuperscript{15}. In addition, DNA sequences within the proglucagon promoter also contribute to tissue specificity. A combination of cell line and transgenic mouse studies have demonstrated that approximately 1.6 kb of the human proglucagon gene 5’-flanking sequences can direct proglucagon transcription to the brain and intestine, whereas additional upstream sequences extending to the first 6kb are required for expression in the pancreatic cells\textsuperscript{16}. Interestingly, within this 6kb region, one conserved intron sequence, designated ECR3, has also been identified to regulate proglucagon expression specifically in the pancreatic alpha cells.

\textbf{1.1.3 Tissue-specific Proglucagon Posttranslational Products}

Proglucagon yields tissue-specific products in the brain, intestine and pancreas. The proglucagon mRNA is identically expressed in these tissues\textsuperscript{10,17}; thus, its specificity is mainly achieved through posttranslational modifications by prohormone convertase (PC) enzymes PC1/3 and PC2\textsuperscript{18} (Fig. 1). Studies have shown that PC1/3 is both necessary and sufficient to complete the proglucagon processing in L-cells to produce glicentin, oxyntomodulin, intervening peptide-2, GLP-1 and GLP-2\textsuperscript{19,20,21}. PC1/3 null mice exhibit
increased levels of intestinal proglucagon accompanied by decreased levels of its cleavage products. PC enzymes responsible for the posttranslational processing of proglucagon in the central nervous system (CNS) are not well established. However, high levels of PC1/3 and PC2 are present throughout the CNS, suggesting a similar processing mechanism to the intestine\(^7\).

Glicentin is the major intestinal peptide that corresponds to proglucagon residues number 1-69 (PG 1-69). Although its exact function is not well defined, studies have shown that it exerts trophic effects on rodent intestinal mucosa, slows down gastric emptying and inhibits extraintestinal invasion of enteric bacteria\(^{22-24}\). Oxyntomodulin (OXM), a cleaved product of Glicentine (PG 33-69), is a dual agonist of the GLP-1 and glucagon receptors\(^{25}\). It promotes satiety, inhibits gastrointestinal secretion and motility, and stimulates pancreatic enzyme secretion and intestinal glucose uptake\(^{23,26}\). Interestingly, injections of OXM in humans caused a significant reduction in weight and appetite as well as increased energy expenditure\(^{27}\).

The two glucagon-like peptides, GLP-1 and GLP-2, are contained in a stretch of proglucagon residues known as the major proglucagon fragment (MPGF, PG 72-160)\(^{28}\). These two peptides are flanked by pairs of basic amino residues, which serve as canonical cleavages sites for PC enzymes. The full length GLP-1 (PG 72-108) is biologically inactive. Further truncation is required to liberate the active form, GLP-1 7-37 (PG 78-108), which acts as a potent stimulator of glucose-stimulated insulin secretion\(^{29}\). Furthermore, it was found that the last residue of full length GLP-1 (PG 108), Gly, serves as a substrate for amidation by the action of peptidylglycine alpha-amidating monooxygenase to generate GLP-1 (7-36)NH\(_2\)\(^{30}\). GLP-1 (7-36)NH\(_2\) is equipotent to GLP-
1 7-37 and has similar metabolism\textsuperscript{31}. It exhibits slightly improved stability towards degradation and is the predominant form in humans\textsuperscript{32,33}. The sequence of naturally occurring GLP-2 corresponds exclusively to PG 126-158 with no sign of further posttranslation modifications\textsuperscript{34}. GLP-2 has a large number of target tissues including the gastrointestinal tract and the CNS. It stimulates cell proliferation and inhibits apoptosis in the intestinal crypt compartment\textsuperscript{35}. It also up-regulates intestinal glucose transport, improves intestinal barrier function, and inhibits food intake, gastric emptying, and acid secretion\textsuperscript{36,37}. In the CNS, GLP-2 improves neuronal proliferation and survival\textsuperscript{38}.

In pancreatic alpha-cells, the proglucagon precursor is mainly processed into glicentin-related polypeptide (GRPP), glucagon, intervening peptide-1, and MPGF\textsuperscript{19}. Glucagon is a hormone that counteracts insulin. It is crucial for maintaining glucose homeostasis in the fasting state by increasing hepatic glucose production via activations of glycogenolysis and gluconeogenesis\textsuperscript{39}. GRPP, intervening peptide-1 and MPGF do not have a known function to date. PC2 is at least partly responsible for the alpha-cell proglucagon processing. Mice that lack active PC2 exhibit deficient proglucagon processing and develop phenotypes that are similar to mice that have a glucagon receptor deletion: mild hypoglycemia, improved glucose tolerance and alpha cell hyperplasia\textsuperscript{40}.

1.1.4 GLP-1 Secretion

Although GLP-1 producing enteroendocrine cells can be found throughout the entire small intestine in pigs, rats and humans, its main synthesizer, the L-cells, are located primarily in the distal ileum and colon\textsuperscript{12}. L-cells are open-type entero-epithelial endocrine cells that have two surfaces: apical and basolateral\textsuperscript{41}. The apical surface faces the
intestinal lumen where it is in direct contact with the luminal nutrients, whereas the basolateral surface faces the neural and vascular tissue. Thus, a variety of nutrient, neural and endocrines factors are involved in regulating GLP-1 secretion from the L-cells. Meal ingestion is the primary physiological stimulus for GLP-1 secretion, which occurs in a biphasic manner in rodents and humans\textsuperscript{42}. The first phase of secretion occurs rapidly after nutrient ingestion (within 15-30 min) and is followed by a prolonged second phase at 90-120 min post-ingestion. Since most L-cells are located in the distal small intestine, it is unlikely that the early phase of GLP-1 secretion is mediated by direct nutrient contact. In fact, placement of glucose or fat in the proximal duodenum could induce an immediate stimulation of L-cells that resembles the early phase of GLP-1 secretion\textsuperscript{43}, suggesting a cross-talk between the proximal and distal small intestine (Fig. 2). The autonomic nervous system has been proposed to mediate this cross-talk as direct activation of the vagus nerve increased fat-induced GLP-1 secretion in rats, whereas bilateral subdiaphragmatic vagotomy completely blocked the secretion\textsuperscript{44}. Both M1 and M2 muscarinic-receptors have been demonstrated to play crucial roles in this process since muscarinic-receptor agonists enhanced GLP-1 release while antagonists inhibited it\textsuperscript{45}. Acetylcholine and gastrin-releasing peptide have been identified as the key neurotransmitters that mediate this proximal-distal loop\textsuperscript{45,46}. In the canines and rodents, the incretin hormone GIP secreted from proximal K-cells also contributes to this early phase of GLP-1 secretion\textsuperscript{43} (Fig. 2).
In contrast to the indirect mechanisms that mediate first phase GLP-1 release, the second phase is believed to occur in response to direct interaction of the distal L-cells with luminal nutrients as they move down the small intestine (Fig. 2B). Indeed, placement of nutrients into the distal ileum stimulates GLP-1 release\textsuperscript{43}. Fat has been proposed to be the more important physiological regulator for GLP-1 secretion as compared to glucose since glucose does not reach distal gut in high concentration. In addition, intestinal somatostatin, neurotransmitter GABA and alpha- and beta-adrenergic agonists, leptin and insulin hormones, have also been demonstrated to modulate GLP-1 secretion through positive and negative feedback loops\textsuperscript{12}. It is of interest that insulin resistance in the L-cells decreases both basal and stimulated GLP-1 release, which may in part account for the reduced GLP-1 secretion observed in obesity and Type 2 Diabetes (T2D) patients.
1.1.5 GLP-1 Metabolism and Clearance

As mentioned previously, GLP-1 presents in multiple forms in vivo. The active forms, GLP-1 7-37 and GLP-1 (7-36)NH₂, are generated from their full-length precursors by the actions of PC1/3\(^4\). Bioactive GLP-1 contains a penultimate alanine residue in position 2, which is a target site for the enzyme dipeptidyl peptidase-4 (DPP-4)\(^4\). DPP-4 is a serine protease that specifically cleaves dipeptides from the amino terminus of proteins, thereby inactivating them. It is widely expressed in multiple tissues and can be found in brush border enterocytes lining the small intestine, as well as endothelial cells positioned directly adjacent to the GLP-1 producing sites. Owing to the rapid actions of DPP-4, bioactive GLP-1 has an extremely short half-life of less than 2 minutes in the circulation. In fact, ex vivo studies have shown that majority of GLP-1 secreted by the L-cells is already degraded into inactive metabolites before even leaving the gut\(^5\). Only about 25% of the newly secreted GLP-1 reaches the systemic circulation in the intact, active form. Conversely, the half-life of GLP-1 could be prolonged by inhibiting DPP-4 in both animals and humans\(^5\). Other than DPP-4, neutral endopeptidase 24.11, a membrane-bound zinc metallopeptidase, has also been demonstrated to degrade GLP-1 via e-terminal cleavage\(^5\). Inactive GLP-1 metabolites are subsequently cleared by the kidney with a half-life of 4-5 minutes\(^5\). Fasting plasma levels of bioactive GLP-1 range between 5 and 10pmol/L in humans and upon feeding, the levels increase approximately 2 to 3 fold depending on the size and nutrient composition of the meal. Postprandial levels of GLP-1 are reduced with obesity and the development of Type 2 Diabetes (T2D)\(^5\).
1.1.6 Biological Actions of GLP-1

1.1.6.1 Pancreas

GLP-1 controls important and diverse physiological functions in multiple organs including the pancreas, brain, gastrointestinal and cardiovascular systems\(^7\) (Fig. 3). Its actions are most extensively studied in the pancreatic beta cells, where it potentiates glucose-stimulated insulin secretion (GSIS), improves glucose sensing, insulin gene transcription and biosynthesis, and increases cell survival and proliferation. The primary effector of GLP-1-enhanced GSIS is cAMP. Upon ligand binding to the GLP-1 receptor (GLP-1R), cAMP accumulates to activate two distinct pathways: PKA-dependent phosphorylation and PKA-independent activation of Epac\(^{5,6,29}\). Through these two pathways, GLP-1 acutely depolarizes the beta cells by increasing ATP synthesis in the mitochondria and promoting closure of the K\(_{ATP}\) channels. It also prevents repolarization of the plasma membrane by closing the Kv channels. As a result, voltage-gated Ca\(^{2+}\) channels are activated, elevating intracellular Ca\(^{2+}\) levels to subsequently increase insulin exocytosis. The glucose-dependency of this GLP-1-induced insulin secretion is not completely understood; however, studies have suggested that K\(_{ATP}\) and Kv channels may be involved\(^7\).

Besides enhancing GSIS, GLP-1 has many other beneficial effects in the beta cells: 1) it increases insulin gene transcription and biosynthesis through cAMP-induced PKA-dependent and independent signaling pathways; 2) it restores glucose sensitivity to previously glucose-resistant beta cells via up-regulation of the glucose transporters and glucokinases; 3) it attenuates ER stress by reducing eIF2\(\alpha\) levels; and 4) it preserves beta
cell mass by either directly activating the GLP-1R to promote beta cell proliferation and inhibit apoptosis or indirectly through the stimulations of insulin, which improves the metabolic environment for beta cell survival \(^{54,55,56}\). These actions of GLP-1 are mediated by multiple signal transduction mechanisms including transactivation of the epidermal growth factor receptor (EGFR), activation of the cAMP/PKA, PI-3K/PKC/Akt and MAPK pathways, and up-regulation of insulin receptor substrate 2 expressions causing the subsequent FoxO1 nuclear exclusion\(^{57,58}\).

GLP-1 also lowers blood glucose by inhibiting glucagon secretion from the alpha cells. This effect may be direct via GLP-1Rs expressed on alpha cells or indirect via stimulation of insulin and somatostatin secretion\(^{59,60}\). Although the exact mechanism whereby GLP-1 inhibits glucagon is unclear, it is known to be glucose-dependent\(^{61}\). In other words, GLP-1-induced glucagonostatic actions will be relieved when blood glucose falls to the normal or hypoglycemic range.

1.1.6.2 Extrapancreatic Tissues: Nervous, Gastrointestinal (GI) and Cardiovascular Systems

GLP-1R signaling in the CNS exerts important regulatory effects on feeding behaviour, gastric motility, blood glucose levels and cardiovascular functions\(^7\) (Fig. 3). There are three sources of central GLP-1: GLP-1 produced within the CNS, peripheral GLP-1 that diffused across the blood-brain barrier and peripheral GLP-1 that communicates with the CNS via sensory afferent vagal neurons\(^62\). Rodent studies have shown that central or peripheral administrations of GLP-1R agonists, Exendin-4, reduced short-term food and water intake, and consequently decreased body weight\(^{63-65}\). These
GLP-1-induced anorectic effects also apply to human subjects. They are likely to be mediated by direct activation of the GLP-1Rs localized at the hypothalamic CNS and vagal afferent nerve fibers. Surgical transection of the brainstem-hypothalamic pathway and bilateral subdiaphragmatic vagotomy completely blocked the GLP-1-induced anorexia in rat. Ablation of the vagus nerve by capsaicin similarly abolished the anorectic effect of peripherally administered exendin-4 in mice confirming a central role for GLP-1 in affecting food intake. In addition to inhibiting food intake, GLP-1R signaling in the murine CNS has also been implicated to modulate whole-body glucose homeostasis by promoting insulin secretion, inhibiting muscle glucose usage and supporting enhanced hepatic glycogen storage under hyperglycemic conditions. Furthermore, central infusion of GLP-1R agonists reduced amyloid-β peptide in vivo and enhanced neurite and PC12 cell differentiation and outgrowth in vitro, indicating that GLP-1 exerts protective actions on neuronal cells. Finally, GLP-1 has been shown to be important for learning and memory since GLP-1R-/- mice showed impaired learning behaviour, which was corrected by hippocampal Glp1r gene transfer.

GLP-1R agonists inhibit meal-induced gastric acid secretion and gastric emptying in the gastrointestinal (GI) system. Attenuation of gastric emptying is important for normalizing postprandial blood glucose elevation especially in T2D patients, as the transit of nutrients from stomach to small intestine is decelerated. When the GLP-1-dependent inhibition of gastric emptying is antagonized by erythromycin in humans, its glucose-lowering effects are also reduced. Mechanisms whereby GLP-1R agonists exert their GI effects are highly complex. Rodent studies have suggested that GLP-1 signaling in the nervous system may play a role since vagal afferent denervation abolished the
GLP-1-induced attenuation of gastric emptying and acid secretion\textsuperscript{7}. Direct mechanisms may also be involved since GLP-1R expression has been reported in the parietal cells of the stomach.

GLP-1 has been demonstrated to exhibit protective effects in the heart\textsuperscript{72}. Intravenous administration of GLP-1R agonists increased systolic and diastolic blood pressure and heart rate in rodents. Although these effects are not observed in humans, a study in T2D patients revealed that GLP-1 infusion improved endothelial function\textsuperscript{73}. Interestingly, GLP-1 treatment has been demonstrated to increase cardiac output, reduce left ventricular end diastolic pressure, and improve myocardial insulin sensitivity and glucose uptake in dog models of cardiomyopathy\textsuperscript{74}. It also reduces infarct size and improves overall performance of isolated rat heart and animal models of myocardial ischemia\textsuperscript{75,76}. GLP-1Rs expressed in the heart and CNS have been suggested to mediate these cardiovascular effects through the activation of cAMP, PI-3K/Akt and p44/42 MAPK\textsuperscript{75}.

\textbf{Figure 3.} Diverse actions of GLP-1.
1.2 Glucagon-like Peptide-1 Receptor (GLP-1R)

1.2.1 Structure and Activation

The GLP-1R is a 463-amino-acid, 7-transmembrane-spanning protein that belongs to the B-class of G-protein coupled receptors (GPCRs). This class of GPCRs also includes the receptors for glucagon, GLP-2 and GIP. The GLP-1R possesses a large hydrophilic, extracellular N-terminus containing one α-helical region and five β–strands, which form two antiparallel β–sheets and six cysteine residues allowing for disulfide interactions. These structures give GLP-1R the classic ‘short consensus repeats’ conserved in the B-family GPCRs that support the N-terminal stability. The large GLP-1R N-terminus is often described as an “affinity trap” that recognizes and binds peptide ligands, as supported by X-ray crystal structures. The binding specifically adopts a widely accepted two-domain model, in which the C-terminus of the ligand interacts with the N-terminus of the receptor for ligand recognition, and the N-terminus of the peptide associates with the receptor core for conformational rearrangement of the receptor structure (Fig. 4). This conformational change elicits a shift in the receptor intracellular loops, which is important for the subsequent G-protein recruitment and signal transduction activity. The two-domain activation model is experimentally supported by studies using chimeric receptors, mutagenesis analysis and photolabile peptide cross-linking. The intracellular C-terminus of GLP-1R, on the other hand, contains three pairs of serine residues that are phosphorylation sites important for agonist-induced homologous and heterologous receptor desensitization and internalization.
1.2.2 Expression, Signal Transduction and Regulation

The GLP-1R is expressed in a wide range of tissues in rodents and humans including alpha, beta and delta cells of the pancreas, the lung, heart, kidney, stomach, intestine, pituitary, skin, ganglion neurons of the vagus nerve, and the CNS\(^7\). It is a pleiotropically coupled receptor capable of signaling through multiple G-proteins: \(\text{G}_{\alpha}\), \(\text{G}_{\alpha q}\), \(\text{G}_{\alpha i}\), and \(\text{G}_{\alpha o}\)\(^8\). Among them, \(\text{G}_{\alpha}\) coupling is the most documented. Upon ligand binding, the GLP-1R undergoes conformational changes that recruit the G-protein complex and catalyze the release of GDP from the \(\text{G}_{\alpha}\) (Fig. 4). \(\text{G}_{\alpha}\) subsequently binds GTP and dissociates from the \(\text{G}_{\beta\gamma}\). Liberated \(\text{G}_{\alpha}\)-GTP activates membrane-bound adenyl cyclase, which in turn catalyzes the conversion of ATP into cAMP. cAMP is the main effector of GLP-1R signaling\(^8\) (Fig. 5) and it turns on both PKA and Epac2 pathways that are responsible for a number of GLP-1 actions. Other than cAMP, the ligand-bound GLP-1R
also activates phosphatidylinositol-3 kinase (PI-3K) through transactivating the epidermal growth factor receptor (EGFR)\textsuperscript{82} (Fig. 5). PI-3K in turn stimulates PKC ζ and Akt/PKB signaling pathways that are responsible for suppressing Kv currents and inducing beta-cell proliferation. In addition, reports have demonstrated that GLP-1R activation could mobilize intracellular Ca\textsuperscript{2+} through phospholipase C\textsuperscript{81}. The effect of GLP-1 on Ca\textsuperscript{2+} mobilization is commonly assumed as a result of Gαq coupling. However, recent studies have shown that GLP-1R activation could induce Ca\textsuperscript{2+} mobilization in GLP-1R-expressing HEK cells without activating Gαq\textsuperscript{83}. Thus, the role of alternative Gα subunits in mediating GLP-1R signaling remains inconclusive. Moreover, GLP-1R activation has been associated with signaling pathways that involve ribosomal S6 kinases and mitogen- and stress-activated protein kinase family of CREB kinases, which at least in part mediate the enhancement of insulin gene transcription via basic region-leucine transcription factors\textsuperscript{81}; as well as pathways that involve β-arrestin-1 and pERK1/2, which aid in promoting beta cell survival. It is apparent that GLP-1R signaling differs according to the cellular context of its expression\textsuperscript{78}. Although this phenomenon is common among all GPCRs, the underlying mechanism is unclear.

The GLP-1R undergoes rapid and reversible homologous and heterologous desensitization in islet cell lines upon ligand stimulation\textsuperscript{84}. Although the exact mechanisms remain elusive, phosphorylation of specific residues in the GLP-1R C-terminus is clearly required. Recruitment of caveolin-1, G protein-coupled receptor kinase and β-arrestin has been suggested to mediate this GLP-1R desensitization even though it is controversial. Recently, computer simulations have suggested that the GLP-1R desensitization undergoes two sequential states\textsuperscript{85}: a first ligand-bound state followed
by a second transition state. Although GLP-1R desensitization is well recognized in vitro, it has not been observed in vivo adding controversies to the field\textsuperscript{84}.

\textbf{Figure 5.} GLP-1 signal transduction pathways in the pancreatic β cell. Signaling in pancreatic β-cells via the classical GLP-1R-coupled G\textsubscript{α}s pathway mediates increases in cAMP to up-regulate PKA and Epac2 (exchange protein activated by cAMP). These pathways increases intracellular Ca\textsuperscript{2+} concentrations through 1) enhancing intracellular Ca\textsuperscript{2+} mobilization, 2) inhibition of K (potassium) channels and 3) acceleration of Ca\textsuperscript{2+} influx through VDCCs (voltage-dependent Ca\textsuperscript{2+} channels). Together, they lead to increased insulin biosynthesis and secretion (green). Activation of proto-oncogene tyrosine kinase src (c-src) and subsequent transactivation of epidermal growth factor receptor (EGFR) aid in increasing PI3K, IRS-2 (insulin receptor substrate 2) and PKB (Akt) to enhance β-cell proliferation (orange). This is also facilitated in part by PKA-mediated increases in MAPKs (mitogen-activated protein kinases), β-catenin and cyclin-D1. Inhibition of caspases, FoxO1 (forkhead box protein O1) and NF-κB (nuclear factor κB), in addition to regulation of CREB (cAMP-response-element-binding protein) and subsequent inhibition of Bcl-2-associated death promoter (BAD), aid in the inhibition of apoptosis (blue), a process also mediated by β-arrestin-1 and the pERK1/2 (phosphorylation of ERK1/2, as well as the IGF-1R/IGF-2 (insulin-like growth factor-1 receptor/insulin-like growth factor2) autocrine loop. ER (endoplasmic reticulum) stress reduction (black) involves the up-regulation of multiple transcription factors, including ATF-4 (activating transcription factor-4), CHOP [C/EBP (CCAAT/enhancer-binding protein)-homologous protein], which inhibits the dephosphorylation of eIF2α (eukaryote initiation factor 2 α). Cross-talk exists between most pathways, including the regulation of the important promoter of insulin gene transcription, synthesis and secretion, Pdx-1 (pancreas duodenum homeobox-1) via both cAMP-dependent and IRS-dependent mechanisms.
1.3 GLP-1 and GLP-1R as Therapeutic Targets for Type 2 Diabetes (T2D)

1.3.1 Therapeutic Potential of GLP-1/GLP-1R

Type 2 Diabetes (T2D) is a metabolic disease marked by chronically elevated levels of blood glucose or hyperglycemia in the context of insulin resistance and deficiency. This disease is often associated with complications including increased risk of cardiovascular disease, lower limb amputation, nontraumatic blindness, cognitive dysfunction and kidney failure. In 2008/2009, almost 2.4 million Canadians are living with T2D (Diabetes in Canada, Public Health Agency of Canada). Estimates suggest that this number will go up to 3.7 million by 2018/2019 if incidence and mortality rates continue at levels seen in the 2008/09 data. Studies have shown that the nutrient-stimulated insulin secretion or incretin effect is largely reduced in T2D patients. This impaired incretin effect is associated with modestly but significantly diminished levels of nutrient-induced GLP-1 secretion. However, the glucoregulatory actions of GLP-1 are preserved in the subjects with T2D. Thus, efforts have been focused on developing “replacement” therapies of GLP-1 and analogs to treat T2D. In fact, many studies have demonstrated that continuous administration of native GLP-1 or three times daily supplementation could significantly reduce meal-related glycemic excursion in the context of increased plasma insulin and reduced glucagon levels. Inhibition of gastric emptying, appetite and food intake have also been observed in subjects infused with GLP-1. Despite the remarkable anti-diabetic pharmacology demonstrated by GLP-1 infusion, its short biological half-life presents a major obstacle limiting its pharmaceutical potential. To combat this shortcoming, numerous efforts have been focused on developing GLP-1 analogs with improved metabolic properties.
1.3.2 GLP-1R Agonists: Exenatide and Liraglutide

To date, two degradation resistant GLP-1R agonists, Exenatide and Liraglutide, have been approved for marketing as treatments of T2D\textsuperscript{78}. These agonists demonstrate an efficacy comparable to insulin treatment but with minimal risk of hypoglycaemia since their actions are glucose-dependent. Exenatide is the synthetic version of GLP-1R agonist exendin-4, a bioactive peptide extracted from the poisonous venom of Heloermatidae lizards\textsuperscript{89}. It is fully efficacious and exerts the same physiologic glucoregulatory actions as native GLP-1\textsuperscript{90,91}. However, Exenatide is a poor DPP-4 substrate and is protected from its degradation\textsuperscript{92}. Instead, it is mainly cleared through glomerular filtration in the kidney\textsuperscript{93}. Thus, Exenatide has a much longer biological half-life of approximately 4 hours as compared to native GLP-1. Pre-clincal trials of Exenatide therapy reported significantly reduced levels of HbA1c (a measure that correlates to blood glucose levels), improved parameters of beta cell function, decreased fasting and postprandial glucose concentrations and loss of weight in T2D subjects\textsuperscript{94-96}. In April of 2005, Exenatide became the first incretin-based therapeutic approved by the United States Food and Drug Administration (FDA) for the treatment of T2D\textsuperscript{78}.

Liraglutide is the second GLP-1 receptor agonist approved to treat T2D. Fatty acid derivatization strategy, which attaches a fatty acid moiety to native GLP-1 facilitating its binding to serum albumin, was used to prolong the action of this molecule in vivo\textsuperscript{97}. Binding to albumin stERICALLY protects Liraglutide from DPP-4 degradation, consequently increasing its half-life to 11-15 hours. Thus, this molecule is suitable for once-weekly administration. Liraglutide received marketing approval in January of 2010 for the treatment of T2D under the brand name Victoza.
1.3.3 DPP-4 Inhibitors

As an alternative to utilizing synthetic GLP-1R agonists as therapy, another option to treat T2D is by attenuating endogenous incretin degradation using DPP-4 inhibitors. Sitagliptin was the first DPP-4 inhibitor approved for clinical use in October 2006 followed by Vildagliptin in Europe and Saxagliptin in the US markets\(^98\). These agents can be used as monotherapy or in combination with other agents. They increase plasma levels of GLP-1 and GIP after food intake, and thus enhance pancreatic GSIS. DPP-4 inhibitors are well tolerated, weight neutral, and are not associated with hypoglycemia\(^99-101\).

1.3.4 Limitations of the GLP-1-based Therapies

There is no doubt that GLP-1-based therapeutics are effective glucose-lowering agents. However, several concerns have been raised regarding their side effects. For example, mild to moderate nausea, vomiting and diarrhea are often associated with the Exenatide therapy\(^102\). The fact that Exenatide needs to be injected twice daily also causes a lot of pain to the patients, possibly resulting in its relatively high withdrawal rate of \(~20\%\)\(^94\). Liraglutide, on the other hand, is suitable for once-weekly dosing. However, gastrointestinal side effects are also common. In addition, Liraglutide treatment may give rise to welling or lump in the throat area, weakness, confusion, increased thirst and signs of infections such as fever\(^102\). Minor gastrointestinal complaints and nasopharyngitis appear to be the most common side effects for the DPP-4 inhibitors.

In contrast to the fairly minor short-term side effects, long-term consequences of the GLP-1-based therapies are far more worrisome. Given GLP-1’s prominent role in increasing beta cell proliferation, pancreatitis or the enlargement of the pancreas may
result from the use of these therapeutics. In fact, studies have shown that Exenatide treatment is associated with a significantly higher reporting rate for acute pancreatitis as compared to the other non-GLP-1 based therapies\textsuperscript{103}, and the FDA issued its first alert for this potential risk in October 2007. Moreover, pancreatitis is well known to predispose individuals to pancreatic cancer. Thus, GLP-1-based treatments may also increase the risk of pancreatic cancer. Furthermore, recent reports have suggested that GLP-1-based therapies may impose an increased risk of thyroid cancer similarly due to its proliferative effects in the thyroid\textsuperscript{102}.

1.4 GPCR Interactomes

1.4.1 GPCR Interactomes Regulate Receptor Signaling

Guanine nucleotide-binding protein-coupled receptors (GPCRs) are the largest class of transmembrane signaling molecules that mediate a myriad of complex physiological processes\textsuperscript{104}. GPCRs are commonly categorized into 5 groups based on conserved structures: class-A rhodopsin-like, class-B secretin-like, class-C glutamate-like, Adhesion and Frizzled/Taste2. GLP-1R, together with the other glucagon-subfamily of receptors, belongs to the B-class of GPCRs\textsuperscript{77}. Despite intensive research effort, little is known about the precise mechanism of GPCR activation and signaling. In fact, an activated GPCR is highly versatile as they interact with a large variety of accessory proteins commonly referred to as interacting proteins or interactors\textsuperscript{105}, and together they form signaling networks known as GPCR interactomes (Fig. 6). The protein interactors predominantly target the C-terminus and sometimes the third intracellular loop of the GPCRs. They serve to modulate a number of GPCR activities including cross-linking to cytoskeleton,
targeting and trafficking between cellular compartments, assembly with other membrane proteins and consequent allosteric regulation and fine-tuning of downstream signaling activities\textsuperscript{106}.

The GPCR interactors can be both transmembrane and cytosolic proteins. Interestingly, some of the transmembrane interactors are themselves GPCRs that form homo and/or heterodimers. For example, the C-terminus of the D5 dopamine receptor has been found to interact with the intracellular second loop of the GABAA receptor gamma2 subunit, which results in a mutually inhibitory interaction between the two receptors\textsuperscript{107}. Similarly, reports have shown that the D1 receptor physically interacts with the NMDA receptor subunits and the GIP receptor interacts with the GLP-1 receptor\textsuperscript{108,109}. Other transmembrane interactors include ion channels, ionotropic receptors and single transmembrane proteins. The discovery of receptor activity modifying proteins (RAMPs) has revealed a new concept of GPCR pharmacology as RAMPs not only assist receptor folding and trafficking, but also contribute directly to the activation and signaling of some B family GPCRs\textsuperscript{110}. There are more than 50 cytosolic GPCR protein interactors that have been identified. They include accessory ion channels subunits, protein kinases, small G proteins, cytoskeletal proteins and adhesion molecules\textsuperscript{105}. It is evident from these studies that the protein interactors to which a GPCR binds at least in part determines its signaling specificity.
1.4.2 Known GLP-1R Binding Proteins

To date, a limited number of interacting molecules have been identified that regulate GLP-1R function. β-arrestin-1, for example, is a cytosolic scaffolding protein that has known interactions with many GPCRs. Interestingly, it has been reported to interact with the GLP-1R in an agonist-dependent manner using bioluminescence resonance energy transfer (BRET) technologies. The GPCR recruitment of β-arrestin is classically associated with an induction of GPCR desensitization. Emerging data has also implicated that β-arrestin may mediate GPCR signaling independent of G-proteins. In fact, β-arrestin recruitment is required for GLP-1-induced cAMP production and insulin
secretion from INS-1 beta cells, even though the underlying mechanisms remain unclear\textsuperscript{114}. Also, studies using MIN6 beta cells have presented β-arrestin as a cAMP independent signaling axis that mediates the prosurvival effects of GLP-1 through the activation of ERK and BAD\textsuperscript{115}. Caveolin-1, another scaffolding protein, has also been shown to interact with the GLP-1R at the plasma membrane\textsuperscript{116}. This interaction is required for the GLP-1R localization to lipid rafts, as caveolin-1 deletion completely abolished GLP-1 binding and activity in vitro. Small ubiquitin-related modifier protein (SUMO), on the other hand, interacts with the GLP-1R to down-regulate receptor trafficking to the plasma membrane, and thus reduces GLP-1 signaling\textsuperscript{117}. Since SUMO expression is increased in mouse islets exposed to high glucose, its interaction with the GLP-1R may in part contribute to the reduced incretin responsiveness in T2D. Moreover, interesting interactions have been characterized within the Glucagon Subfamily Receptors. For example, GIPR heterodimerizes with the GLP-1R in a ligand-dependent manner\textsuperscript{109}. This interaction decreased GLP-1-induced β arrestin-1 recruitment to the GLP-1R and consequently shifted calcium influx, suggesting a potential allosteric regulation between the GSRs.

1.5 Rational and Hypothesis of Thesis

GLP-1R agonists regulate diverse and important functions in multiple organs, especially the pancreas. Despite the numerous efforts that have been invested to delineate the complex signaling cascades of GLP-1R activation, the exact mechanism remains largely unknown. It is well accepted that GLP-1R signaling differs according to the cell type where it is expressed in; a common phenomenon shared among many GPCRs. Also, studies have shown that GPCR interaction partners have profound effects at modulating
receptor signaling and as discussed, a few GLP-1R interacting proteins have already been revealed suggesting that the GLP-1R may have many other unknown interactors. Therefore, I hypothesize that the GLP-1R has undiscovered interacting proteins capable of modulating the receptor function, specifically in pancreatic beta cells. These interaction proteins may at least in part account for the increasingly diverse actions of GLP-1 and the complex nature of GLP-1R signaling. Also, manipulation of these interacting proteins may serve as novel strategies to enhance GLP-1/GLP-1R activity and thus, its therapeutic potential. Furthermore, revealing and studying the GLP-1R interactome may provide valuable insights into understanding B-family GPCR signaling in general, advancing the field of GPCR research.
Chapter 2: Identification of GLP-1R interacting proteins that restrain the receptor activity

2.1 Introduction

Glucagon-like peptide 1 (GLP-1) is an incretin hormone mainly produced and secreted from the enteroendocrine L-cells upon feeding\(^7,118\). Acting through its native glucagon-like peptide 1 receptor (GLP-1R), it controls diverse and important physiological functions. GLP-1 is best known to enhance glucose-stimulated insulin secretion (GSIS) from the pancreatic beta cells. Interestingly, this activity is maintained in patients suffering from Type 2 Diabetes (T2D)\(^119\), a metabolic disorder characterized by chronically elevated blood glucose. Therefore, therapeutic strategies targeting GLP-1 and GLP-1R have been developed to treat T2D. In addition to augmenting GSIS, GLP-1 improves glucose sensing; insulin gene transcription and biosynthesis; and survival and proliferation of the beta cells\(^7,118\). GLP-1 also has complex actions in extra-pancreatic tissues, including being a potent satiety factor that signals the central nervous system to inhibit food intake, decreasing gastric emptying and modulating gastric acid secretions. Moreover, GLP-1R agonists have been demonstrated to protect myocardial cells and improve heart function\(^72\).

GLP-1R, together with other members of the glucagon subfamily of receptors, glucagon-like peptide 2 receptor (GLP-2R), glucose-dependent insulinoitropic polypeptide receptor (GIPR), and glucagon receptor (GCGR), belongs to the secretin-like class B family of guanine nucleotide-binding protein-coupled receptors (GPCRs)\(^77,78\). These receptors signal primarily through the activation of a G\(_s\)-protein complex and the second messenger cAMP upon ligand binding. A wide array of accessory proteins have
been shown to interact directly with the GPCRs to form functional protein interactomes\textsuperscript{105,106,120}. These accessory proteins modulate multiple receptor activities such as receptor folding, subcellular trafficking and intracellular signaling. To date, a limited number of interacting molecules have been identified to regulate the GLP-1R function. β-arrestin-1, for example, is a scaffolding protein shown to be required for the stimulation of GLP-1-induced cAMP production and insulin secretion from INS-1 beta cells\textsuperscript{114}. Caveolin-1, another scaffolding protein, interacts with the GLP-1R to stabilize its localization at the plasma membrane\textsuperscript{116}. Interesting interactions within the GSR subfamily have also been characterized. For example, GIPR heterodimerizes with GLP-1R to decrease GLP-1-induced β arrestin-1 recruitment to the GLP-1R, suggesting a potential allosteric regulation between the GSRs\textsuperscript{109}. These findings collectively raise the possibility that the GLP-1R has many other undiscovered accessory proteins capable of modulating the receptor function. It is, therefore, important to reveal the GLP-1R interactome, which may account for the increasingly diverse actions of GLP-1 and the complex nature of GLP-1R signaling.

The membrane based split-ubiquitin yeast two hybrid (MYTH) system is a modified yeast two-hybrid screen that does not require the protein of interest to be expressed in the nucleus\textsuperscript{121,122} (Fig. 7). Therefore, it allows the study of full-length proteins in situ at the cell membrane. This screen is designed based on the finding that an ubiquitin protein can be split into two halves: Cub and Nub. By homologous recombination, the membrane protein of interest is fused with the Cub linked with an artificial transcription factor (TF) as bait, while the interactor protein candidate is fused with the NubG as prey. NubG is a mutated form of Nub to prevent spontaneous reconstitution with the Cub. The bait and
prey vectors are sequentially transformed into a competent yeast host, *Saccharomyces cerevisiae* strain THY.AP4. Interaction of bait and prey proteins will bring Cub and NubG into close proximity, leading to reconstitution of the two to form a pseudo-unbiquitin, which acts as a substrate for ubiquitin-specific protease (UBP). UBP consequently cleaves the pseudo-unbiquitin to release the TF from the bait construct. The liberated TF translocates into the nucleus to activate downstream reporter genes for nutrient (HIS, ADE2) and colorimetric (LacZ) selections. As a result, yeast colonies expressing positive interactions can survive Adenine and Histidine depleted SD-WL media (SD-WLAH) and will appear blue. MYTH has already been used to identify protein interactors of GPCRs, including the *Caenorhabditis elegans* D2-like dopamine receptor, the mammalian M3 muscarinic receptor and mu-opioid receptor, and the human frizzled 1 receptor. It has also been useful for studying interactions of other membrane bound proteins and transporters such as the glucose transporter.

In the present study, several novel interacting proteins have been identified to construct the first GLP-1R interactome. Using MYTH, 38 interacting candidates were revealed for the receptor in absence of ligand stimulation. These interactors were validated by co-immunoprecipiation and immunofluorescence analyses. I continued to investigate the functional effects of these interactors and found that many significantly attenuated GLP-1R signaling when over-expressed in Chinese Hamster Ovarian (CHO) cells. Three beta cell expressing membrane-bound interactors, solute carrier family 15 member 4 (SLC15A4), amyloid beta A4 precursor-like protein 1 (APLP1) and adaptor-related protein complex 2 mu 1 subunit (AP2M1), were further selected for individual knock down in MIN6 beta cells using siRNAs. GLP-1-induced insulin secretion was
significantly enhanced when these genes were silenced, strongly suggesting that these interactor proteins attenuate GLP-1R activity.

Figure 7. Membrane-based split-ubiquitin yeast two-hybrid system (MYTH). (A) The MYTH system is composed of bait and prey proteins. The bait protein is a transmembrane protein of interest fused with a C-terminal ubiquitin (Cub) and an artificial transcription factor comprising LexA and VP16 (TF). The prey protein is a potential interactor fused with an N-terminal ubiquitin (NubG). (B) On the interaction of the bait and prey proteins, Cub and NubG reconstitute a pseudo form of ubiquitin, which leads to the ubiquitin-specific protease (UBP) dependent proteolytic release of the downstream TF. The liberated TF translocates into the nucleus and induces the expression of HIS3, ADE2, and LacZ reporter genes in the yeast. HIS3 and ADE2 allow the yeast to grow on synthetic dropout minus tryptophan/leucine/adipine/histidine (SD-WLAH) plates and β-galactosidase (LacZ) expression is required for colorimetric selection.

2.2 Material and Methods

2.2.1 Construction of yeast and mammalian cell expression GLP-1R plasmids

The coding sequence of GLP-1R was amplified from human islet cDNA by PCR using Pfu Turbo DNA polymerase (Stratagene Invitrogen, Burlington, Ontario, Canada). The GLP-1R was cloned into a yeast expression pCWW-ste vector fused with C-terminal ubiquitin (Cub) and artificial transcription factor LexA-VP16 (TF) (Dualsystems Biotech, Zurich, Switzerland) by homologous recombination. The human fetal brain cDNA library
was cloned into a pPR3-N vector fused with point-mutated N-terminal ubiquitin (NubG). This prey NubGx library was commercially made by Dualsystems Biotech (Zurich, Switzerland). All of the constructs were validated by sequencing. Alternatively, the coding sequence of the receptor was cloned into mammalian cell expression vectors pcDNA3.1D/V5-His-TOPO and pcDNA3.1D/FLAG-TOPO for functional validation. The cDNAs of identified interactors on the other hand were cloned into pcDNA3.1D/3XHA vector. Plasmids were prepared using GenElute plasmid Midiprep kit (Sigma, Oakville, Canada)

2.2.2 Cell culture and Transfection

HEK293T cells and CHO cells were maintained in DMEM (Gibco Invitrogen, Burlington, ON, Canada), containing 10% FBS and 100 U/ml penicillin (P/S) at 37°C in 5% CO₂. MIN6 cells were maintained in DMEM containing 15% FBS, 100U/ml P/S and 1.7μL beta-mercaptoethanol per 500ml. These cells were transfected with the GLP-1R, interactors and siRNAs via Lipofectamine 2000 following the manufacturer’s protocol (Invitrogen, Burlington, Ontario, Canada). A human GLP-1R expressing CHO cell line (RC2-CHO) was established by selection using 1mg/mL G418 (Gibco, Invitrogen, Burlington, Ontario, Canada) and was maintained in 0.1mg/mL G418.

2.2.3 MYTH Screening

Membrane based split ubiquitin yeast two-hybrid (MYTH) screen is designed based on the finding that an ubiquitin protein can be split into two halves: Cub and Nub (Fig. 7)\textsuperscript{127}. By homologous recombination, Glp-1r is fused with the Cub-TF as bait, while the interactor protein is fused with non-interacting NubG (for MYTH screening) or
spontaneously reconstituting NubI (for control experiments) as prey. During MYTH screening, Cub and NubG reconstitute when the bait and prey proteins interact, leading to proteolytic cleavage of the TF by an ubiquitin-specific protease. The liberated TF then translocates into the nucleus to activate reporter genes for nutrient and colorimetric selections. MYTH was performed as previously described\textsuperscript{127}. Briefly, bait and prey vectors described above were sequentially transformed into the competent host \textit{Saccharomyces cerevisiae} strain THY.AP\textsubscript{4} following Gietz and Woods’s method\textsuperscript{128}. The yeast two-hybrid selection was achieved on synthetic dropout tryptophan/leucine/adenine/histidine deficient (SD-WLAH) plates. All positive colonies were inoculated in yeast. The plasmids harboring interactor sequences were extracted and amplified in Escherichia coli, and purified using mini-prep kits (Qiagen, Toronto, Ontario, Canada). All the plasmids were sequenced and the sequences were further blasted in the NCBI database for potential interactor candidates. To validate the interaction, each interactor was individually transformed back into the yeast overexpressing the bait (bait dependency test). Interactors which passed the test were screened through various online databases to determine expression at the gene, protein and mRNA levels in specific tissues (Human Protein Atlas (HPA) www.proteinatlas.org), cellular localization (Human Protein Reference Database (HPRD) www.hprd.org), functional category and known physiological and/or pathophysiological relevance (PubMed Central www.ncbi.nlm.nih.gov/pubmed and BioGPS www.biogps.org) and the Beta Cell Biology Consortium (www.betacell.org) to understand the possible role of the interactors in pancreatic beta cells.

\textbf{2.2.4 Immunofluorescence}
Yeast and CHO cells were grown onto poly-L-lysine glass slide and fixed for 30 min in 4% paraformaldehyde at room temperature. The yeast slides were washed in 1 ml SK solution (1.8mM K$_2$HP0$_4$, 1.7mM KH$_2$PO$_4$, 3.6mM sorbitol, pH7.5) and then re-suspended in 100-200 µl SK solution. The CHO cells were washed three times with PBS and then permeabilized with 0.1% Triton™X-100. In both cases, blocking was performed with 3% BSA in PBS for 30 min at RT in a humidified chamber, followed by counterstaining with rabbit anti-VP16 (1:200 dilution; Sigma, Oakville, Ontario, Canada), rabbit anti-Flag (1:250 dilution; Cell Signaling, Whitby, Ontario, Canada), and rat anti-HA (1:500 dilution; Roche, Mississauga, Ontario, Canada) antibodies for 1 hour at room temperature. Secondary antibodies used were Alexa Fluor®488 Goat Anti-Rabbit and Alexa Fluor®546 Goat Anti-Rat IgG (Invitrogen, Burlington, Ontario, Canada). Slides were imaged using spinning disk confocal microscopy and Pearson’s correlation coefficient (PCC) was calculated using Volocity 3D Image Analysis Software (Perkin Elmer, Vaughan, Ontario, Canada). PCC has a range of +1 (perfect correlation) to -1 (perfect negative correlation), with 0 denoting the absence of a relationship.$^{129}$

**2.2.5 Co-immunoprecipitation and immunoblotting**

Interactor overexpressed RC2-CHO cells were washed with ice-cold PBS and harvested in lysis buffer (1%digitonin, 5mM imidazole, 1 Protease Inhibitor Cocktail Tablet per 10mL, PBS). The cell lysate was centrifuged and the supernatant was extracted and incubated with Ni$^{2+}$ beads that were equilibrated with the wash buffer (0.1% digitonin, 5mM imidazole, 1 Protease Inhibitor Tablet, PBS) for 1h at 4°C. The precipitation lysate was washed and eluted in PBS containing 1X SDS loading buffer. The precipitated proteins were analysed by western immunoblotting. 40µl of the
precipitated proteins from each sample was loaded and separated on a 10% BioRad TGX gel and transferred using the BioRad Transfer system (BioRad, Mississauga, Ontario, Canada). 1:2500 Anti-V5 (Invitrogen, Burlington, Ontario, Canada) and 1:5000 anti-HA primary antibodies (Covance, Montreal, Quebec, Canada) and HRP conjugated mouse secondary antibody were used for detection. Signal was detected by chemiluminescence (GE Healthcare, Inc., Vaughan, Ontario, Canada) and images were acquired using the Kodak image station 4000pro (Carestream Health, Inc, Vaughan, Ontario, Canada). The same procedure was used for anti-FLAG tag CO-IP, in which anti-FLAG M2 Affinity beads (Sigma, Oakville, Ontario, Canada) were used instead of Ni$^{2+}$ beads.

2.2.6 GLP-1 or Forskolin Stimulated cAMP Formation

Intracellular cAMP content was measured using homogeneous time resolved fluorescence (HTRF) technology according to the manufacturer’s protocol (Cisbio US, Bedford, Massachusetts, United States). Briefly, interactor transfected RC2-CHO cells were plated onto 384-well cell culture plates at a confluence of 3000cells/well. Cells were preincubated with DMEM (0.5%BSA) for 30min and then incubated with indicated concentrations of GLP-1 (Abcam, Toronto, Ontario, Canada) or forskolin (Sigma, Oakville, Ontario, Canada) in DMEM (0.5%BSA, 1µM 3-isobutyl-1-methylxanthine) for 30min. After treatment, cells were incubated with lysis buffer containing cAMP-D2 and cryptate conjugate tracers for 1 hour and counted using PHERAsstar Plus microplate reader (BMG Labtech, Guelph, ON)

2.2.7 Insulin Secretion Assay, Reverse Transcription PCR and Quantitative PCR
MIN6 cells transfected with siRNAs (Dharmacon, Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States) were equilibrated in Krebs-Ringer Bicarbonate Buffer buffer (in mM, NaCl 115, KCl 5, NaHCO3 24, CaCl2 2.5, MgCl2 1, HEPES 10, BSA 0.1%) for 1.5h and stimulated with indicated concentration of glucose or GLP-1 for 1h. The supernatant was collected for insulin measurement using HTRF following the manufacture’s protocol (Cisbio US, Bedford, Massachusetts, United States). Reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR) was performed as previously described. Briefly, cells were lysed and total RNA was extracted using an RNeasy Plus Kit (Qiagen, Toronto, Ontario, Canada) and converted to cDNA using a reverse transcription kit (Sigma, Oakville, Ontario, Canada). Power SYBR green PCR master mix was used for qPCR following the manufacturer’s protocol (Applied Biosystems, Invitrogen, Burlington, Ontario, Canada). The software used was ViiA™ 7 Real-Time PCR System (Applied Biosystems, Invitrogen, Burlington, Ontario, Canada). The sequences of all the qPCR primers were listed in Table 2.

2.2.8 Statistics

Paired t-tests and two-way ANOVA were performed to determine statistical significance between intracellular cAMP and insulin measurements. P<0.05 was considered statistically significant.

2.3 Results

2.3.1 Identification of GLP-1R interacting proteins by MYTH

GLP-1R is an integral membrane receptor, thus, MYTH screen was chosen to detect its interacting proteins in a membrane environment. The interactor candidates were
identified from a NubG-fused human fetal brain cDNA prey library because GLP-1R is expressed in the CNS, where it mediates important functions as I have discussed previously. To generate a bait construct compatible with MYTH, human GLP-1R cDNA was subcloned into a yeast-expression pCCw-ste vector fused with the Cub and LexA-VP16 transcription factor (TF) tag at the C-terminus. Proper expression and localization of the receptor at the yeast plasma membrane was validated by immunofluorescence (Fig. 8A). The functionality of the receptor was tested by subcloning the same cDNA into pcDNA3.1, a mammalian-expression vector. GLP-1R overexpressed HEK293T cells showed a dose-dependent increase of cAMP accumulation upon GLP-1 stimulation (Fig. 8B), indicating that the cloned GLP-1R was biologically functional.

Protein interaction capability within the MYTH system and self-activation of the bait constructs were verified by NubG/NubI test using non-interacting plasma membrane and ER resident proteins, Ost1p and Fur4p, as positive (pOst1-NubI and pFur4-NubI) and negative (pOst1-NubG and pFur4-NubG) prey controls. Co-transformation of the control prey plasmids and the bait did not affect yeast growth on normal SD-WL (Trp and Leu deficient) media (Fig. 8C). However, when the co-transformants were plated on selection Adenine and Histidine depleted SD-WLAH media, yeast growth was only observed with the NubI-fused proteins but not with the NubG-fused proteins (Fig. 8C). These results indicated that the GLP-1R was properly transported through the secretory pathway (positive pOst1-NubI, ER), expressed at the plasma membrane (positive pFur4-NubI) and not self-activating in the yeast (negative pOst1-NubG and pFur4-NubG). Incubation with a competitive inhibitor of the His3 reporter gene, 3’-aminotriazole (3’AT) (10mM), was chosen to minimize inherent leaky His3 reporter expression. At this concentration,
growth with the negative NubG control prey was entirely abolished but not with the positive NubI control prey (Fig. 8C). Before library screening, the GLP-1R expression was also confirmed in the human fetal brain by RT-PCR (Fig. 8D).

Three independent assay screens using the unliganded hGLP-1R bait and fetal brain cDNA library prey have been performed, covering approximately 31% of the library with an average of 1.81 x 10^6 clones/screen. A bait dependency test was carried out to validate the identified interactions in yeast. In the test, interactors were individually transformed back into the GLP-1R expressing yeast and subjected to selection. To pass the test, at least 2 of 3 colonies of the bait-prey co-expressing yeast must grow on the selection media in three independent trials. TSPAN13, SYNGR3 and NRGN represented interactors that passed the test, while NPTN failed (Fig. 8E). Using this strategy, 38 novel interactors were identified for the GLP-1R (Table 1, Fig. 9). Searching for the proteins in various databases (NCBI, HPA, HPRD, SymAtlas, BCC) revealed 7 of them to be associated with cell signaling, 6 with metabolism (energy, protein, nucleic acids, carbohydrates), 5 with cell proliferation and development, 5 with transmembrane transportation, 4 with trafficking, endo and exocytosis, 3 with protein processing and degradation, 2 with transcription regulation, 2 with immune response and 4 with other or unknown functions (Fig. 9). Thus, a diverse group of novel receptor interactor has been identified.
Figure 8. MYTH screening using an unliganded Cub- and TF (LexA-VP16)-fused human GLP-1R as bait and a NubG-fused human fetal brain cDNA library as prey. (A) Proper expression of the GLP-1R in yeast. Yeast cells transformed with the human GLP-1R were fixed, permeabilized, and probed for VP16. Immunofluorescence indicated the proper expression of the constructed bait vector in yeast. Bar, 10 µm. (B) Characterization of the GLP-1R in HEK293T cells. GLP-1-stimulated cAMP accumulation was measured in HEK293T cells transfected with the bait (n=3). (C) NubG/NubI self-activation test. The bait vector harboring GLP-1R was cotransformed into yeast with control constructs of Ost1p (target endoplasmic reticulum) or Fur4p (target plasma membrane) fused to NubG or NubI. The cotransformants were selected on either tryptophan/leucine (SD-WL) or tryptophan/leucine/adenine/histidine (SD-WLAH) depleted synthetic dropout plates with increasing concentrations (10 mM, 25 mM, 50 mM) of 3'-aminotriazole. Each row of the plate showed a serial dilution of the yeast cells from 1 to 10⁶. (D) GLP-1R expression in the human fetal brain. GLP-1R was amplified from the human fetal brain tissue by RT-PCR. Human islets were used as a positive control. (E) Validation by the bait-dependency test. The interactors identified from MYTH were cotransformed with the Cub-TF human GLP-1R into yeast and the cotransformants were selected on SD-WLAH plates with 10 mM 3'-aminotriazole to validate receptor-protein interactions.
Fig. 9: GLP1-R interactome identified by MYTH. MYTH revealed 38 interactors for the GLP-1R. Among them, 7 are associated with cell signaling, 6 with metabolism (energy, protein, nucleic acids, carbohydrates), 5 with cell proliferation and development, 5 with transmembrane transportation, 4 with trafficking, endo and exocytosis, 3 with protein processing and degradation, 2 with transcription regulation, 2 with immune response and 4 to have other or unknown functions.
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Table 1. List of MYTH identified GLP-1R Interactors
2.3.2 Validation of the interaction between GLP-1R and interactors in mammalian cells

2.3.2.1 Coimmunoprecipitation

It was important to examine whether the MYTH-identified receptor-protein interactions occur in the context of mammalian cells, so co-immunoprecipitation (CO-IP) was performed. A stable human GLP-1R (V5-6XHistidine tagged) overexpressing CHO cell line (RC2) was created for these studies. The 38 MYTH-identified GLP-1R interacting candidates were subcloned into HA-tagged pcDNA3.1 vectors and independently introduced into the RC2 cells by transient transfection. Protein complexes were precipitated using Ni\(^{2+}\) beads targeting the His-tag on the GLP-1R, and western blotting was performed to detect the interactors. For each of the interacting proteins, intensity of the elute/precipitate band was quantified and expressed as a ratio to the lysate band (E:L ratio). The E:L ratio was used to estimate the binding affinity of interactors to the GLP-1R; the closer the E:L ratio of a particular interactor that was to 1, the stronger its interaction with the GLP-1R. Among the 38 proteins, 6 had high E:L ratios (E:L≥0.7; SVOP, AP2M1, MFSD5, GABBR2, SLC15A4, ELOVL1), 19 had intermediate E:L ratios (E:L=0.3-0.7; CEND1, C18orf32, GPR37, STMN3, SYNGR3, CFI, ACSF3, PIGG, CAND2, PAQR6, SLC31A2, HPN, C5orf32, IFITM3, PH4, CD81, TMEM147, APH1A, STOML2), 10 had low E:L ratios (E:L=0.1-0.3; MRPL22, APLP1, MYL6, FDFT1, C14orf166, ATP6V0B, ERP29, ATP13A1, TSPAN13, NUCKS1) and only 3 had insignificant E:L ratios and failed to interact with the GLP-1R (E:L≤0.1; ARFRP1, STMN1, NRGN) (Fig. 10A,B). To confirm these results, I transiently co-transfected Flag-tagged GLP-1R and selected interacting candidates into a native CHO cell line and
performed co-immunoprecipitation using anti-Flag tag antibodies (Fig. 11). Since the E:L ratios obtained from anti-FLAG tag CO-IP were similar to that of using Ni$^{2+}$ beads, it is confident that the interacting molecules bind to the bait. These results provided validation of the MYTH system for identifying GLP-1R-protein interactions.

**2.3.2.2 Immunofluorescence**

Subcellular distribution of selected interactors was examined using confocal laser scanning microscopy. In CHO cells, Flag-tagged GLP-1R and HA-tagged interactors were transiently co-expressed and immuno-stained for their respective tags. The Pearson’s Correlation Coefficient (PCC) was calculated to quantify the degree of co-localization for each interactor with the receptor. CHO cells overexpressing either the GLP-1R or the interactor, NRGN, alone were used to control for the background fluorescence and antibody specificity (Fig. 12A-12B). All of the 13 interactors showed a degree of co-localization with the GLP-1R, with a PCC ranging from 0.21 to 0.89 (Fig. 12F). Interactors such as MRPL22 and ACSF3 had PCC values that fell below the level of NRGN (0.27), the protein that failed to co-precipitate with the GLP-1R, were deemed to have marginal co-localization with the receptor.
Figure 10. Validation of the interaction between GLP-1R and the MYTH-identified interactors in RC2-CHO cells. HA-tagged interactors were independently transfected into the human GLP-1R (V5-His tagged) expressing RC2-CHO cells. CO-IPs were performed using Ni²⁺ beads targeting the His-tagged GLP-1R and Western blots were performed to detect the precipitants. Intensities of the protein bands in the elute were expressed as a ratio to the bands in the lysate (E:L) to estimate the binding strength for each interactor. (A) Western blots (anti-HA) showing protein interactors with high (**), medium (*), low (−), and insignificant E:L ratios. L, lysate; W3, wash 3; E, elute. (B) Plot of E:L ratios estimating binding strength. Three independent CO-IPs were performed for each interactor.
Fig. 11: Validation of the interaction between Flag-tagged hGLP-1R and the MYTH identified interactors in CHO cells. Flag-tagged GLP-1R and HA-tagged interactors were co-transfected into CHO cells. CO-IP was performed using Anti-Flag beads targeting the Flag-tagged GLP-1R and western blots were performed to detect the precipitants. A. Western-blots (anti-HA) showing protein interactions with high (***), medium (**), low (*) and insignificant E:L ratios. L = Lysate, W3 = Wash 3, E = Elute. B. Intensity of the protein band in the elute was expressed as a ratio to the band in the lysate (E:L) to estimate the binding strength for each interactor. Two independent CO-IPs were performed for each interactor.
Fig. 12: Co-localization analysis of the GLP-1R and selected interactors by immunofluorescent imaging. CHO cells were over-expressed with the Flag-tagged GLP-1R and HA-tagged interacting proteins and stained for their respective tags. Pearson’s correlation coefficient was calculated to indicate the degree of co-localization. A. CHO cells overexpressing the GLP-1R alone were used as a control for non-specific anti-HA (interactor) binding. Bar=26µm B. CHO cells overexpressing the NRGN alone were used as a control for non-specific anti-Flag (GLP-1R) binding. Bar=26µm C-E. CHO cells coexpressing the GLP-1R and selected interactors with high, intermediate and low PCCs. Two representative images were presented for each interactor protein. Bar=26µm. F. Plot of Pearson’s coefficient indicating degree of colocalization. PCC = +1 (perfect correlation); PCC = 0 (absence of a relationship). Interactors with PCCs lower than NRGN were deemed to have low co-localization with the GLP-1R (n=25-30 cells from 3 independent studies).
2.3.3 Interactor proteins reduced the GLP-1R function

2.3.3.1 GLP-1R interactors modulate GLP-1-stimulated cAMP accumulation in CHO cells

cAMP is a principal signaling molecule generated by the binding and activation of B-class GPCRs\textsuperscript{77,78}. Therefore, I quantitatively assessed GLP-1R signaling by measuring cAMP accumulation. CO-IP-validated interactors were expressed in RC2 cells under basal (0 GLP-1) and stimulated (0.25nM GLP-1) conditions. The dose of 0.25nM of GLP-1 was chosen for the assay because it is lower than the EC50 concentration 0.5nM\textsuperscript{131}, and thus would not saturate the receptor. Of the 35 validated proteins, 17 showed no significant effects while surprisingly, the other 18 all reduced GLP-1-induced cAMP accumulation (GABBR2, GPR37, PAQR6, ACSF3, ELOLV1, STMN3, SLC15A4, SLC31A2, SVOP, APLP1, AP2M1, TMEM147, ERP29, HPN, IFITM3, C15orf32, TSPAN13 and PIGG)(Fig. 13). Interestingly, the majority of these suppressive interactors clustered in the functional groups of signal transduction (Group A, 3 out of 7), transmembrane transportation (Group D, 3 out of 5) and intracellular trafficking (Group E, 3 out of 4). No interactor was however found to significantly increase cAMP accumulation. To ensure the observed suppressive effects were occurring at the level of GLP1-R activation and not simply related to the overexpression of the interactors, I treated the cells with forskolin, which bypasses the receptor to stimulate cAMP formation. SVOP, TMEM147 and GPR37 significantly attenuated forskolin-induced cAMP (Fig. 14) indicating an intrinsic ligand-independent suppression. These three proteins were therefore not further studied, although these observations are of interest.
Fig. 13: The effect of interactors on GLP-1-induced cAMP. RC2-CHO cells over-expressing the individual interactors were stimulated with 0 and 0.25nM of GLP-1. The corresponding cAMP accumulation was measured by HTRF and NRGN was used as the negative control. The interactors were organized into their function groups: A=Signal Transduction, B=Metabolism, C=Cell Proliferation & Development, D=Transportation, E= Trafficking, F=Protein Processing, G=Transcription Regulation, H=Immune Response, I=Others & Unknown. (n=4), * = p<0.05, ** = p<0.01, *** = p<0.001
Fig. 14: Elimination of interactors that have an intrinsic suppressive effect on cAMP production. RC2-CHO cells over-expressing the individual interactors were stimulated with 0 and 5µM of forskolin. The corresponding cAMP accumulation was measured by HTRF. NRGN was used as the negative control (n=4), * = p<0.05
2.3.3.2 Overexpression of selected GLP-1R interactors showed no significant effect on GLP-1 stimulated insulin secretion in MIN6 beta cells

The GLP-1R is expressed in pancreatic beta cells, where it is known to augment insulin secretion in a glucose-dependent manner upon ligand binding. Therefore, I continued to examine whether these interactors modulate GLP-1R function in the beta cells. I first confirmed GLP-1R expression in the mouse insulinoma (MIN6) beta cells by RT-PCR and qPCR (Fig. 15A-B). Next, I overexpressed selected GLP-1R interactors into the MIN6 cells and measured their respective effects on GLP-1-induced insulin secretion at 20mM glucose. Surprisingly, no significant change in insulin secretion was observed consequent to the overexpressions (Fig. 16).

![Fig 15: Validation of GLP-1R and interaction protein expressions in MIN6 cells. A. GLP-1R was amplified from MIN6 by RT-PCR. B. Endogenous expression level of the GLP-1R and the interactors was assessed by qPCR. Interactor transcript quantities were normalized to the level of Kcnj11, a gene that codes for a subunit of beta cell K_{ATP} channel. Proteins with transcript levels of 90% Kcnj11 or higher were deemed to have high expression in the MIN6.](image)
Reducing expression of selected GLP-1R interactors enhances GLP-1-stimulated insulin secretion in MIN6 beta cells.

Overexpression of the interacting proteins reduced GLP-1-induced cAMP accumulation in CHO cells but did not affect insulin secretion in MIN6. Thus, I continued to examine whether knocking down these interactors could elicit an effect. I first validated endogenous expression of the interactors, which were originally identified from the fetal brain cDNA library, in MIN6 (Fig. 15B). Of the 15 interactors that attenuated GLP-1R signaling in CHO, 7 (SLC15A4, APLP1, AP2M1, TSPAN13,
ELOVL1, ERP29, STMN3) were expressed in MIN6 at levels higher than or comparable (at least 90%) to the gene, *Kcnj11*. *Kcnj11* encodes Kir6.2, subunit of the well characterized beta-cell adenosine triphosphate dependent potassium (K\(_{\text{ATP}}\)) channel that is integral for the regulation of insulin release\(^{132}\); therefore, I used its transcriptional level as a reference for choosing the proteins of interest. Since the GLP-1R functions at the cell membrane, I selected 3 membrane-bound interacting proteins, SLC15A4, APLP1, AP2M1 out of the 7 candidates for knock down studies.

MIN6 cells were treated with either non-targeting siRNA (scrambled control) or siRNAs targeting each of the three interactors. The transcript expression level of each interactor was knocked down by over 85% as compared to the control (Fig. 17A). I stimulated the tested cells with 10nM of GLP-1 at both low and high glucose conditions (0mM and 20mM) and measured insulin secretion (Fig. 17B). In the scrambled control, GLP-1 treatment increased insulin secretion by 22.0±4% at 20mM glucose (Fig. 17C) but not at 0mM. This is expected, because GLP-1 response is known to be glucose dependent. When the three interactor proteins, SLC15A4, APLP1 and AP2M1 were individually knocked down, GLP-1-induced insulin secretion was significantly increased to 56.0±10% (p<0.001), 49.2±7% (p<0.001) and 47.9±8% (p<0.05) respectively at 20mM glucose (Fig. 17C). However, in the absence of GLP-1, there was no difference in insulin secretion at low or high glucose in any group (Fig. 17B). Taken together, these data strongly suggest that the MYTH-identified interactors attenuate ligand-stimulated GLP-1R activity in beta cells at high glucose.
Fig. 17: Knocking down selected interactors enhanced GLP-1R function in MIN6 cells. SLC15A4, APLP1 and AP2M1 were individually knocked down in MIN6 cells using siRNA. Cells were treated with 10nM of GLP-1 at low (0mM) and high (20mM) glucose and incubated for 1 hour. Cell medium was collected for insulin measurement by HTRF. MIN6 cells transfected with non-targeting siRNA were used as a control (Scramble).

A. qPCR showing efficient knock-down of SLC15A4, APLP1 and AP2M1 by siRNA as compared to the control.

B. GLP-1 induced insulin secretion at low and high glucose.

C. Percent increase of insulin secretion upon GLP-1 treatment at high glucose (HG, 20mM). (n=4), * = p<0.05, *** = p<0.001
Chapter 3: General Discussion

3.1 Summary of Findings

Recent studies have revealed a large range of accessory proteins that interact directly with the intracellular domains of the GPCRs, forming functional protein units or interactomes\textsuperscript{105}. In this study, we screened a human fetal brain cDNA library to produce the first GLP-1R interactome using the MYTH system (Fig. 9). Bait dependency test in yeast and CO-IP and immunofluorescence analysis in mammalian CHO cells were used to validate the protein-receptor interactions. Bioinformatic analysis revealed the interactors to be involved in signal transduction, metabolism, cell growth and trafficking. Using cAMP and insulin secretion assays, we further showed that a subset of the interacting proteins attenuated the receptor function. Thus, inhibiting these interactors may serve as a novel strategy to enhance GLP-1R activity.

3.2 MYTH Identified GLP-1R Interactome

Using the MYTH screen, we have for the first time identified an interactome for the GLP-1R (Fig. 9). GLP-1R is a GPCR best known to maintain global glucose homeostasis and promote pancreatic beta cell proliferation. Thus, it is interesting to find the majority of GLP-1R interacting candidates to be involved in cell signal transduction, metabolism, proliferation and development (Fig. 9). However, none of these proteins has been previously recognized as an interactor of the GLP-1R. The inactive state of GLP-1R during the MYTH screen may in part explain why well-known interaction partners of the GPCRs, such as G-proteins, are not identified. Also, some interaction proteins may be neglected from the screen as false negatives as discussed previously. Furthermore, three
MYTH screens only covered about 31% of the human fetal brain cDNA library in this study, leaving the remaining 69% unstudied.

Of the 38 MYTH identified GLP-1R interaction proteins, 7 are functionally clustered into cell signaling. These proteins include small GTPases such as ARFRP1. GTPases are a family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP) and they play essential roles in mediating GPCR signaling. Thus, it is interesting to reveal such GTPases as novel interacting partners of the GLP-1R. GLP-1 has marked effects on nutrient metabolism; therefore, it is not surprising that its receptor interacts with metabolic factors. Six interactors identified from the screen are involved in metabolism, which include ELOVL1 and ACSF3. ELOVL1 is a condensing enzyme that catalyzes the synthesis of both saturated and monounsaturated very long chain fatty acids, whereas ACSF3 activates the breakdown of complex fatty acids (UniProt). Revealing these factors suggests an action of GLP-1 on fat metabolism. Proteins that are involved in cell differentiation and proliferation have also been identified to interact with the GLP-1R, which corresponds to its role in enhancing beta and neuronal cell survival and proliferation. CEND1, for instance, is known to enhance pig neuroblastoma cell differentiation in vitro and may be involved in neuronal differentiation in vivo.

Furthermore, in agreement with previous findings, this study showed that GPCRs could interact with one another. In fact, we have identified multiple GPCRs in the GLP-1R interactome including GABBR2 and GPR37.

3.3 Limitations of MYTH

The split-ubiquitin MYTH is a novel, high-throughput method for studying protein-protein interactions (PPIs) developed by Dr. Igor Stagljar’s laboratory at the University of
Toronto\textsuperscript{127}. It is often the method of choice for analyzing full-length integral membrane proteins such as GPCRs because it is not restricted by their hydrophobicity. Positive interactors are easily identified in MYTH through growth selection, colorimetric or fluorescent phenotype and prey plasmid DNA is readily accessible for sequencing. Furthermore, MYTH can detect interacting proteins expressed at very low abundance in vivo. However, every technology has its limitations. One shortcoming of the MYTH assay is that the bait, GLP-1R in this case, is not activated during the screens because peptide ligands cannot penetrate through the yeast cell wall to stimulate receptors expressed on the plasma membrane\textsuperscript{136}. Thus, MYTH does not detect protein complexes that would be recruited by agonist activation. In order to study ligand dependency of the receptor interactome, additional innovative strategies will be required to mimic the receptor activation process.

Artifacts within the prey interactor datasets are also limiting factors of MYTH. These artifacts include some bona fide interactors that are not picked up by the screen, so-called “false negatives”. Bona fide interactors may arise from a number of possibilities: 1) failure of subcellular trafficking of these interacting proteins to the receptor; 2) disruption in the sequence of protein trafficking, protein modification (i.e. phosphorylation) and sequential assembly and disassembly, that collectively lead to the eventual functional multimeric receptor complex; 3) lack of required post-translational modification in the yeast host organism; 4) improper folding of the Nub fusion prey protein; or 5) interference of key binding domains of the receptor or interactor by fused ubiquitin tags\textsuperscript{137}. Moreover, partial cDNA clones that make up the cDNA prey libraries may also lack all the domains necessary to interact with the bait receptor as they normally would at
full-length. Finally, these proteins may be underrepresented or even absent from the cDNA library used for yeast two hybrid (YTH) screening.

False positives are also common in yeast two hybrid (YTH) screens\textsuperscript{138}. There are two types of false positives. The first type results from the binding of study protein to the bait protein of interest only in the context of YTH assay, but not in a true physiologic setting. An explanation for this phenomenon is that this study protein contains certain requisite recognition sequences for the bait, but does not normally interact with the bait protein in normal cellular conditions. The second type of false positive occurs when the study protein induces TF activity independent of any meaningful PPI. This phenomenon may be a consequence of overexpression of bait proteins, a process that can inadvertently cause endogenous expression of ubiquitin tag leading to self-activation. Furthermore, this artificial process can induce plasmid rearrangements or copy number changes that generate auto-activators, or alter reporter genes in a manner that causes constitutive expression\textsuperscript{137}. Finally, MYTH is restricted to identifying only binary interactions, leading to an underrepresentation of those proteins that interact only within entire protein complexes.

### 3.4 Validations of the MYTH Identified Interactors

As mentioned above, artefacts are common in MYTH screening. Thus, I have performed a series of validation assays to ensure the accuracy of the screen. The bait dependency test was first used to validate the interactors (Fig. 8E). This test is very useful at eliminating false positives generated by MYTH, however, it should be noted that it is not 100% accurate and is performed in yeast but not mammalian cells. To further validate the interactor proteins in a mammalian setting, CO-IPs were performed using a stable-
hGLP-1R expressing RC2-CHO cell line. Thirty-five of the 38 GLP-1R interactor candidates co-precipitated with the receptor, highlighting the dependability of MYTH screen to examine mammalian protein-protein interactions for membrane-spanning proteins. It should be noted however that the CO-IPs were carried out in a model system, and overexpressing some proteins may promote artificial interactions. Since globally, the proteins identified interacted with the receptor with differing strengths (Fig. 10B), and some completely failed to bind the bait (NRGN, STMN1 and ARFRP1), I am confident that these CO-IP interactions were not simply artifacts of overexpression. The complementary anti-Flag CO-IP (Fig. 11) and co-localization (Fig. 12) results also confirmed the positive binding between GLP-1R and the MYTH-identified interactors. However, it would be ideal to show endogenous interactions, which would require good quality antibodies for the native proteins. Unfortunately, it has been reported that commercially available GLP-1R antibodies are not specific, which is consistent with my data as the anti-GLP-1R antibody (Abcam 39072) failed to identify the overexpressed GLP-1R in CHO cells (Fig. 18A). To address the interactors, I tested three antibodies that are commercially available (Sigma SAB3500044 for AP2M1, Sigma SAB2102167 for SLC15A4 and Thermo Scientific PA5-23720 for GABBR2) for western blotting (Fig. 18B) and immunofluorescence (Fig. 18C). Unfortunately, the results are again negative for overexpressed interactors with the antibodies in question.
Fig 18. Commercially available antibodies are not specific. A. Anti-GLP-1R antibody was tested by western blotting. Lysates of untransfected CHO cells were loaded in lane 1; lysates overexpressed with Flag-tagged GLP-1R were loaded in lane 2 and 3. Anti-Flag antibody was used as the positive control. B. Anti-AP2M1, anti-APLP1 and anti-GABBR2 antibodies were tested by western blotting. In each blot, lysates of untransfected MIN6 were loaded in lane 1; lysates overexpressed with the respective HA-tagged interactors were loaded in lane 2 and 3. Anti-HA antibody was used as the positive control. C. MIN6 cells were transfected with HA-tagged GABBR2 and HA-tagged SLC15A4 in the first two columns and stained with anti-HA and anti-GABBR2/anti-SLC15A4 antibodies respectively. In the last column, untransfected MIN6 cells were stained with anti-GABBR2 or anti-SLC15A4 antibodies for endogenous proteins. Bar=26µm
3.5 MYTH Identified GLP-1R Interaction Proteins Restrained the Receptor Activity

Interestingly, 15 out of the 35 CO-IP validated GLP-1R interactors attenuated cAMP accumulation in the RC2 cells upon GLP-1 stimulation, but none enhanced it (Fig. 13). Since the initial MYTH screening was performed using an unliganded inactive GLP-1R, it is possible that the identified interactors suppress receptor activation. These putative interactors may act to attenuate receptor activity, stabilizing the GLP1-R in a low-activity state in absence of ligand binding. Although there is no direct evidence supporting this idea, knocking down SLC15A4, APLP1 and AP2M1 significantly enhanced GLP-1-stimulated insulin secretion from the MIN6 cells (Fig. 17B, C). I did not observe any significant change to the basal insulin secretion in absence of GLP-1 at both low and high glucose when the interactors were knocked down individually (Fig. 17B). This further suggests a functional redundancy and compensation among the protein interactors, which act as a whole to keep the GLP-1R quiescent in its unliganded state. The inability to detect interactors capable of enhancing cAMP accumulation may also be explained by effects on the cAMP-independent signaling cascades of the GLP-1R. For example, stimulation of the GLP-1R in pancreatic beta cells has been reported to activate pathways that involve transactivation of the EGFR and subsequent PI3 kinases. The identification of interactors that work through such pathways will require employing different screening assays. It was to my surprise that overexpression of the protein interactors did not affect GLP-1-induced insulin secretion in MIN6 cells (Fig. 16). This could be explained by the endogenous expression of these proteins in MIN6, which may be sufficient to keep the
receptor inactive. Thus, increasing the amount of the interactors did not give rise to further effects.

3.6 Potential Functional Relevance of the Interactor Candidates

3.6.1 SLC15A4

The interactors that we identified may modulate GLP-1R function through distinct mechanisms. SLC15A4 is a proton oligopeptide co-transporter that transports free histidine, protons and certain di- and tri-peptides across cell membranes\textsuperscript{139}. Protonation has long been known to affect multiple GPCR properties, including the rate of ligand-induced conformational change and the receptor-mediated G-protein activation\textsuperscript{140}. By co-transporting protons and peptides, SLC15A4 may couple the GLP-1R activity to the extracellular nutrient state through modulating the local pH. Interestingly, a single nucleotide polymorphism in the \textit{SLC15A4} gene has been reported to associate with the development of T2D\textsuperscript{141}, supporting the regulatory role of this transporter on GLP-1R. The exact in vivo function of SLC15A4 is however largely unknown and its investigation is beyond the scope of this study.

3.6.2 APLP1

APLP1 is a membrane-associated glycoprotein that belongs to a highly conserved amyloid precursor protein gene family. It has been previously reported to interact with the \(\alpha_{2A}\)-adrenergic receptor, a \(G_i\) coupled GPCR, and the voltage gated calcium channel \(\text{Ca}_2.3\)\textsuperscript{142,143}. In both cases, a considerable shift of the receptor/channel localization from the plasma membrane to the intracellular compartments was observed. Clathrin is suggested to play a role in this APLP1-mediated re-localization because APLP1 contains a highly conserved NPxY motif that is known to mediate endocytosis via clathrin coated
pits. APLP1 may similarly induce GLP-1R endocytosis via the same mechanism, which would explain the decrease in GLP-1R activity observed in our study. The fact that I show AP2M1, a subunit of the Adaptor Protein 2 (AP2) complex known to regulate GPCR recycling via clathrin\textsuperscript{144}, also attenuated GLP-1R signaling further supports this idea (Fig. 13, Fig. 17B,C). Interestingly, localization of the GLP-1R shifted from the plasma membrane to the intracellular space in CHO cells when AP2M1 was over-expressed (Fig. 12D).

Upon intracellular cleavage of APLP1 by proteases in the secretase family, a cytoplasmic fragment is released, which may act as a transcriptional activator to mediate apoptosis\textsuperscript{145}. Similarly, islet amyloid polypeptide (or amylin) can induce apoptotic cell death of pancreatic β-cells, which has been implicated in the development of type 2 diabetes\textsuperscript{146}. Most interestingly, Needham et al. (2008) demonstrated that APLP1 and APLP2 double knocked out mice exhibit both hypoglycemia and hyperinsulinemia, which strongly suggests that these proteins play a role in regulating glucose homeostasis\textsuperscript{147}. Thus, it is possible that GLP-1R-APLP1 interactions contribute to the cytoprotective and glucoregulatory effects of GLP-1.

3.7 Protein Interactors as Allosteric Regulators of the GLP-1R and Their Therapeutic Potential

The interacting proteins that are identified in this study act allosterically, that is at sites distinct to the endogenous ligand. From a therapeutic perspective, targeting the receptors allosterically have several major advantages including: 1) enhanced receptor subtype selectivity; 2) the ability to simultaneously bind to the receptor with the endogenous ligand (restoring physiologically relevant temporal control); 3) induce a new repertoire of
receptor conformations and therefore influencing receptor activity; and 4) in particular for peptide-activated receptors, introducing the potential for oral administration\textsuperscript{80}. At present, very few allosterically acting ligands have been identified for the GLP-1R including the Novo Nordisk compounds 2-(2′-methyl)thiadiazolylsulfanyl-3-trifluoromethyl-6,7-dichloroquinoxaline (compound 1) and 6,7-dichloro-2-methylsulfonyl-3-t-butylaminoquinoxaline (compound 2)\textsuperscript{148}, the latter of which demonstrates glucose-dependent insulin release via the GLP-1R\textsuperscript{148,149}. My thesis study implicates that interacting partners of the GLP-1R can regulate the receptor activity allosterically; thus, they may serve as new target sites for drug development. Since all of the interactors that are identified restrained the receptor signaling, antagonization of these proteins may serve as novel strategies to enhance the insulintropic effects of GLP-1R.

\textbf{3.8 Alternative Proteomic Screening Strategies – AP-MS}

Many biochemical approaches have been developed to detect PPI including affinity precipitation and mass spectroscopy (AP-MS), protein chip technology using tagged gene arrays, protein fragment complementation in mammalian cells, and fluorescence (FRET) and bioluminescence resonance energy transfer (BRET) approaches\textsuperscript{150,151}. AP-MS is a well-established high-throughput technology comparable to MYTH for allowing studies of full-length GPCR as the bait\textsuperscript{152}. This approach isolates interacting proteins by affinity pull-down using an affinity-tagged bait protein of interest. Thus, AP-MS can be performed in mammalian cells providing an in vivo environment for interactions. The isolated protein complexes are separated by gel electrophoresis and identified by mass spectrometry. The advantage of AP-MS is that it allows the detection of higher order interactions as it captures entire protein complexes\textsuperscript{153}. Also, only one fused artificial tag
is present in AP-MS, which lessens the chance of interference with PPI. However, the strategy also has potential limitations: 1) the difficulty in distinguishing between specific and nonspecific interactors; 2) the unreliable quantification of changes in the abundance of protein complexes or their components; 3) weak hydrostatic/transient interactions between proteins may be disrupted by harsh biochemical conditions during the purification step; 4) often is difficult to identify low-abundance interacting proteins; and 5) overexpression of bait protein can lead to artifact or false-positive interactions\textsuperscript{154}. Currently, a post-doctoral fellow in my lab has used AP-MS and purified the GLP-1R interactome in both CHO and MIN6 cells. Very interestingly, he observed differential interaction profile for the GLP-1R in the stimulated and non-stimulated state (Unpublished). However, further validation tests are required.

3.9 Alternative Proteomic Screening Strategies – FRET/BRET

FRET is a useful tool for analyzing interactions between two molecules\textsuperscript{155}. Here, the interacting partners are labeled with two fluorophores, the “donor” and the “acceptor” that share overlapping emission/absorption spectrum, respectively. When the two partners interact, the fluorophores are brought into close proximity, allowing for an energy transfer from the donor to the acceptor in the form of photons through nonradiative dipole–dipole coupling. The efficiency of this energy transfer or FRET efficiency can be used to determine the exact distance between the two binding partners and quantify the interaction. One common fluorophore pair is a cyan fluorescent protein (CFP) – yellow fluorescent protein (YFP) pair. Both of these fluorophores are color variants of the green fluorescent protein (GFP). A major limitation of FRET is the requirement for external illumination to initiate the energy transfer, which can lead to photobleaching and/or
background noise due to direct excitation of the acceptor. To avoid this drawback, BRET has been developed\textsuperscript{156}. BRET uses a bioluminescent luciferase rather than CFP to produce an initial photon emission compatible with YFP. One major advantage of FRET/BRET over other tools is that they allow for real-time analysis of protein-protein interactions in live cells. Also, FRET/BRET is suitable for assaying interactions in different subcellular compartments or specific organelles of the native cell. To date, BRET technology has already been used extensively to study oligomerization between B1 class of GPCRs including the secretin receptor, the glucagon receptor, and the glucagon-like peptide-1 and -2 receptors (GLP-1R and GLP-2R)\textsuperscript{157}. 
Chapter 4: Future Directions and Conclusion

4.1 Future Directions

4.1.1 Assess the effects of interactors on MIN6 apoptosis and proliferation.

As I have mentioned previously, GLP-1R activation not only enhances insulin secretion but also promotes proliferation and inhibits apoptosis of the pancreatic beta cells. Thus, it would be of interest to examine whether overexpressing or knocking down selected interactor candidates of interest may affect these two parameters using MIN6. Viability assays such as the caspase assay can be used.

4.1.2 Validate PPI using Bioluminescence/Fluorescence Resonance Energy Transfer Strategies (BRET/FRET)

Up to this stage, all of the PPI validations, CO-IP or IF, were performed in overexpression model systems statically in vitro. It would be ideal to examine endogenous interactions between the GLP-1R and interacting proteins in actual cellular conditions. However, due to the quality issues of the commercially available antibodies that I have mentioned previously, it is technically difficult to address this question at the current stage. Assessing PPI using BRET/FRET is an interesting alternative as it allows the study of PPI temporally and spatially in living cells. Using BRET/FRET, ligand or glucose stimulatory effects on GLP-1R-protein interactions can also be assessed in real-time.

4.1.3 Assess the functional effects of GLP-1R interactors in mouse or human islets.

All of the functional assays that have been performed were carried out in cell lines, CHO or MIN6. Thus, it would be of interest to examine whether these effects similarly
occur in the context of mouse or human islets. Specifically, interacting proteins can be introduced or knocked down from the islets using viral approach with ShRNAs. cAMP and insulin secretion assays can then be performed.

4.1.4 Assess the mechanisms by which the inhibitory effects of SLC15A4, APLP1 and AP2M1 were mediated.

Using cAMP and insulin secretion assays, I have shown that SLC15A4, APLP1 and AP2M1 restrained the GLP-1R activity in MIN6 cells. It is important to discover the mechanisms that underlie these inhibitory effects. As I have discussed previously, APLP1 and AP2M1 are trafficking proteins that may affect the localization of GLP-1R via clathrin-mediated endocytosis. Thus, ligand-binding assays can be performed to quantify the amount of GLP-1R expression on the plasma membrane under APLP1 and AP2M1 overexpression or knock down conditions. Clathrin can be further knocked down to assess whether it is indeed involved in this trafficking event.

4.1.5 Construct and functionally characterize an islet expressing GLP-1R interactome

As mentioned above, GPCR interactomes are tissue specific. In other words, the interaction proteins that are revealed from the fetal brain tissue may not represent the interactome profile in the pancreatic beta cells. Given the beneficial effects that GLP-1 has on insulin biosynthesis and secretion in the beta cells, it would be ideal to identify an islet-specific GLP-1R interactome using an islet prey library. An islet is ~60% beta-cells, thus, it will likely to contain a full complement of GLP-1R interacting proteins for normal beta-cell function.
4.2 Conclusion

This present study is the first example of an interaction screen using the full-length GLP-1R. I show that the unliganded GLP-1R interacts with a large and diverse group of proteins. The functional assays suggest that these interactors act collectively to attenuate GLP-1R activity. Taken together, my data sheds light on the allosteric regulation of GLP-1R, which may also apply to other GSRs or GPCRS in general. Furthermore, some of the newly identified GLP-1R accessory proteins may allow for selective manipulation of the GLP-1R activity.
<table>
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<tr>
<th>Gene Name</th>
<th>Sequence</th>
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<tr>
<td>mPAQR6 (qPCR)</td>
<td>Forward: 5'-CTATGCAGCACTCACATGC-3'&lt;br&gt;Reverse: 5'-CTTGCTGAACCCGGGTATT-3'</td>
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<td>mERP29 (qPCR)</td>
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</tr>
<tr>
<td>mSTMN3 (qPCR)</td>
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<td>Forward: 5'-GCCCTGGAGGCGAGTTCAG-3'&lt;br&gt;Reverse: 5'-CTTGCTGAACCCGGGTATT-3'</td>
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hGLP-1R (Homologous Recombination for Bait Construct) | Forward: 5'-CGGCCAGCGCTTTAATTAAGGCCGCCGTGCCCCTGGCACTCAGTTGACATGACTGCAATGGCTGATGGCCACAGCACGACGAC interviewing |
| mGLP-1R (RT-PCR, Fig. 2D) | Forward: 5'-CTGCCCTTCTGTGTTTCTC-3'<br>Reverse: 5'-ATGAGCAGGATGGGATCTCCTG-3' |
| mGLP-1R (RT-PCR, Fig. 8A) | Forward: 5'-CTGCCCTTCTGTGTTTCTC-3'<br>Reverse: 5'-ATGAGCAGGATGGGATCTCCTG-3' |

Table 2. Primer Sequences for RT-PCR and qPCR
## Abbreviations Footnote

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AP-MS</td>
<td>affinity precipitation and mass spectroscopy</td>
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<tr>
<td>AP2</td>
<td>Adaptor protein 2</td>
</tr>
<tr>
<td>AP2M1</td>
<td>Adaptor-related protein complex 2 μ1 subunit</td>
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<tr>
<td>APLP1</td>
<td>Amyloid β precursor-like protein 1</td>
</tr>
<tr>
<td>ATF-4</td>
<td>activating transcription factor-4</td>
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<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
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<td>c-src</td>
<td>proto-oncogene tyrosine kinase src</td>
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<td>CHO</td>
<td>Chinese hamster ovarian cells</td>
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<tr>
<td>CHOP</td>
<td>C/EBP (CCAAT/enhancer-binding protein)-homologous protein</td>
</tr>
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<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO-IP</td>
<td>Coimmunoprecipitation</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response-element-binding protein</td>
</tr>
<tr>
<td>Cub</td>
<td>C-terminal ubiquitin</td>
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<tr>
<td>E:L ratio</td>
<td>The ratio of the intensity of the elute to the lysate band</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>eIF2α</td>
<td>eukaryote initiation factor 2 α</td>
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<td>Epac2</td>
<td>exchange protein activated by cAMP</td>
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<td>endoplasmic reticulum</td>
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<td>Ex-4</td>
<td>exendin 4</td>
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<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
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<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
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<td>GI</td>
<td>gastrointestinal system</td>
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<td>glucose-dependent insulinotropic polypeptide</td>
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<td>glucagon-like peptide 1</td>
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<td>glucose-stimulated insulin secretion</td>
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<td>Glucagon subfamily of receptors</td>
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<td>Homogeneous time-resolved fluorescence</td>
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<td>insulin receptor substrate 2</td>
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<td>mitogen-activated protein kinases</td>
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<td>MIN6</td>
<td>Mouse insulinoma β cells</td>
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<td>MPGF</td>
<td>major proglucagon fragment</td>
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<td>MYTH</td>
<td>membrane-based split ubiquitin yeast two-hybrid assay</td>
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<td>Point mutated N-terminal ubiquitin</td>
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<td>prohomrone convertase 1/3, 2</td>
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<td>PCC</td>
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<td>pancreas duodenum homeobox-1</td>
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<td>phosphoinositide 3-kinase</td>
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<td>protein kinase A</td>
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<td>protein kinase B</td>
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<tr>
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<td>protein kinase C</td>
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<td>PPI</td>
<td>protein-protein interaction</td>
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<td>receptor activity modifying proteins</td>
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<td>RC2-CHO</td>
<td>Human GLP-1R expressing CHO cell line</td>
</tr>
<tr>
<td>REEP2</td>
<td>Receptor accessory protein 2</td>
</tr>
<tr>
<td>SD-WL</td>
<td>Synthetic dropout minus tryptophan/leucine</td>
</tr>
<tr>
<td>SD-WLAH</td>
<td>Synthetic dropout minus tryptophan/leucine/adenine/histidine</td>
</tr>
<tr>
<td>siRNAs</td>
<td>small interfering RNAs</td>
</tr>
<tr>
<td>SLC15A4</td>
<td>Solute carrier family 15 member 4</td>
</tr>
<tr>
<td>T2D</td>
<td>type-2 diabetes</td>
</tr>
<tr>
<td>TF</td>
<td>Artificial transcription factor comprised of LexA-VP16</td>
</tr>
<tr>
<td>UBP</td>
<td>Ubiquitin-specific protease</td>
</tr>
<tr>
<td>VDCCs</td>
<td>voltage-gated calcium channels</td>
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</table>
References


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