

## Research Article

# Screening for Insulin-Like/Mimetic Drugs Using Lower Eukaryotes

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## Abstract

It has generally been assumed that hormones and the corresponding intra- and intercellular signal transduction pathways and mechanisms have evolved exclusively during course of the evolution of vertebrate endocrine organs, implying a rather recent origin. However, there is good experimental evidence for (i) the expression of hormones and hormone-binding proteins resembling those of vertebrates in fungi and yeast, (ii) functional responses of lower eukaryotes to mammalian hormones and (iii) the existence of components of insulin-like and mimetic signaling pathways as well as their coupling to G-protein coupled receptors and metabolic pathways, such as lipolysis and endoplasmic reticulum stress, in lower eukaryotes, in particular in *Neurospora crassa* and *Saccharomyces cerevisiae*. Data will be presented that the naturally occurring or recombinant expression of insulin-like/mimetic signaling pathways in lower eukaryotic cells may be useful as model systems for future drug screening and discovery efforts.

**Keywords:** Diabetes; Drug Screening; Endoplasmic Reticulum Stress; Glucagon-like Peptides; G-protein-coupled Receptors; Insulin; Lipolysis, *Saccharomyces cerevisiae*

## 1. Introduction: Models for the discovery of insulin-mimetic and anti-diabetic drugs

The yeast *Saccharomyces cerevisiae*, as a simple and well-understood eukaryotic model organism, offers direct means to unequivocally assess processes that couple ligand binding to receptor activation, formation of transient intermediates, and downstream signaling to the molecular targets of the activated receptor. For example, events proximal to insulin-binding at the receptor can be traced to distal events to up to the terminal effector systems, such as dephosphorylation of GS or phosphorylation of ribosomal proteins. Furthermore, the interactions between multiple mutations, their suppressors, and ultimately, between the molecular participants, in total, in an insulin-activated response cascade can be readily tested in yeast. The ease of biochemical, cell biological, genetic and molecular biological manipulation has made *S. cerevisiae* an excellent model for the study of metabolic control in eukaryotic cells. In fact, yeast can be considered as the preferred model eukaryotic organism for the following reasons: I) Many cellular processes are conserved between yeast and higher eukaryotes, including (i) metabolic and energy-generating pathways, (ii) signal transduction pathways, (iii) protein targeting to specific subcellular locations, e.g. secretion, biogenesis of plasma membranes and mitochondria, (iv) peptide maturation, e.g. signal sequence cleavage, prohormone cleavage at Lys-Arg residues, (v) protein modification, e.g. Ser/Thr/Tyr phosphorylation, N- and O-glycosylation, acylation, attachment of GPI anchors, (vi) nuclear

processes, e.g. transcription, RNA processing and polyadenylation. II) Many proteins of yeast are very close orthologs to mammalian counterparts, and their functional replacement by the latter has been shown for some yeast components (Table 1), signal transduction components such as protein kinases and phosphatases (Table 2). III) Yeast represents the best understood eukaryotic organism at the genetic level under the control of a compact and simple genome with few introns, repetitive sequences and non-coding elements. IV) Yeast is ideally suited for both classical and molecular genetic manipulation with many powerful tools introduced during the past five decades, including (i) high and low copy number plasmid vectors, (ii) strong and inducible promoters, (iii) high transformation efficiency ( $> 10^5/\mu\text{g}$  of DNA), (iv) gene replacement in one week, (v) identification and cloning of genes acting along a pathway with the methods of functional complementation, genetic suppression, synthetic lethality etc.

## 2. G-Protein Coupled Receptors as Targets

G-protein-coupled receptors (GPCRs) as transmembrane proteins represent components of intracellular signaling cascades which transduce information from the cell surface into the cell interior in course of agonist binding and the resulting activation of heterotrimeric G-proteins. GPCRs together with their corresponding elements of downstream signaling have been found to operate in all eukaryotes from yeast to mammals [1] and to share pronounced sequence similarities between yeast and mammals (Table 3). It has recently been recognized that in mammalian cells a complex interplay

and mutual cross-talk exists between the insulin signaling cascade and the signaling pathways directed by GPCRs. It depends on the type of GPCR whether insulin action is modulated in a positive or negative fashion and thus insulin sensitivity becomes increased or diminished (i.e. insulin sensitizing or desensitizing) [2–8]. The basis of the successful screening for anti-diabetic drugs is formed by modeling of the activation of the G-protein-coupled class of human receptors in yeast. The successful coupling of human receptor activation to the mating pathway of yeast has been demonstrated [9–11] which should thereby enable the screening for both agonists and antagonists, depending on the needs. The putative targets encompass known members of the GPCR class which are assumed to be involved in the pathogenesis of T2D, among them the glucagon receptor, glucagon-like-peptide 1 receptor (GLP-1) and a variety of  $\alpha$ - and  $\beta$ -adrenergic receptor subtypes, such as muscarinic acetylcholine receptors and various receptors of the brain and nervous system. Significant applications to many other therapeutic areas can be expected where GPCR members play a role.

**Table 1.** Structural comparison of some signal transduction proteins/genes between mammals and yeast with regard to %identity of amino acids. As indicated some mammalian genes can restore the function of the disrupted homologous gene in *Saccharomyces cerevisiae*.

| Mammalian Protein                                | Yeast Gene        | % Identity | Functional Replacement of Yeast Homolog |
|--|-------------------|------------|---|
| Cell Division Protein Kinase                     | <i>CDC28</i>      | 60         | yes                                     |
| MAP Kinases ERK1, 2                              | <i>FUS3/KSS1</i>  | 54         | yes                                     |
| JNK1   | <i>HOG1</i>       | 52         | yes                                     |
| Protein Kinase C <sub><math>\gamma</math></sub>  | <i>PKC1</i>       | 50         | no                                      |
| Osmotic Stress Kinase p38                        | <i>HOG1</i>       | 52         | yes                                     |
| ser/thr Phosphatase PP1                          | <i>PP1</i>        | 81         | no                                      |
| p21Ras   | <i>RAS1/RAS2</i>  | 50–85      | yes                                     |
| GTP-binding Protein G <sub>p</sub>               | <i>CDC42</i>      | 80         | yes                                     |
| G <sub><math>\alpha</math></sub>                 | <i>GPA1</i>       | 40–50      | yes                                     |
| G1 Cyclins                                       | <i>CLN1, 2, 3</i> |            | yes                                     |
| Phospholipase C <sub><math>\gamma</math></sub> 2 | <i>PLC1</i>       | 27–53      | no                                      |
| Transcription factor Egr1/2                      | <i>Msn4</i>       |            | yes                                     |
| Triglyceride Lipase ATGL                         | <i>Tgl4</i>       |            | yes                                     |

The experimental procedure involves (i) targeted mutagenesis of the endogenous GPCR and G <sub>$\alpha$</sub>  genes, (ii) covalent linkage of the human GPCR to yeast G <sub>$\alpha$</sub>  by gene fusion to overcome the lack of recognition specificity between the human GPCR and the yeast G <sub>$\alpha$</sub> , and (iii) mutagenesis of the G <sub>$\alpha$</sub>  domain and selection for functional coupling to ensure efficient interaction (Figure 1). The functional assays designed to enable convenient measurements and clear-cut read-outs are typically, but not exclusively, based on (i) growth arrest and morphological change to “shmoos”, (ii) auxotrophic selection, i.e. activation of the GPCR leads to expression of an essential gene which will support growth in minimal medium, (iii) color development, i.e. activation of the GPCR leads to expression of  $\beta$ -galactosidase, (iv)

fluorescence, i.e. activation of the GPCR leads to expression of *A. victoria* green fluorescent protein (Figure 2).

**Table 2.** Some selected yeast homologs of mammalian protein kinases and phosphatases with established function in signal transduction.

| Protein Kinase or Phosphatase  | Yeast Homolog                   |
|--|---------------------------------|
| AMP-Activated Protein Kinase (AMPK)  | <i>SNF1</i>                     |
| Calcineurin B (catalytic subunit, Ca <sup>2+</sup> -dependent serine/threonine phosphatase)  | <i>CNA1, CNA2 (CMP1, CMP2)</i>  |
| Calcineurin B (regulatory subunit, Ca <sup>2+</sup> -dependent serine/threonine phosphatase) | <i>CNB</i>                      |
| Calmodulin Kinase II   | <i>CMK1, CMK2</i>               |
| cAMP-Dependent Protein Kinase (catalytic subunit)  | <i>TPK1 (SRA3), TPK2, TPK3</i>  |
| cAMP-Dependent Protein Kinase (regulatory subunit)   | <i>BCY1 (SRA1)</i>              |
| Cell Cycle Kinase (p34CDC2)  | <i>CDC28</i>                    |
| Cell Division Kinase (CDK3)  | <i>CDC28</i>                    |
| Cyclin Dependent Kinase (CDK2)   | <i>CDC28</i>                    |
| Cytosolic Tyrosine Phosphatase (MEG2)  | <i>SEC14</i>                    |
| Glycogen Synthase Kinase 3   | <i>MDS1</i>                     |
| Lipopolysaccharide-activated Kinase (JNK1, c-jun amino-terminal protein kinase)              | <i>HOG1</i>                     |
| MAP Kinase Kinase (MKK or MEK)   | <i>STE7, MKK1, MKK2, PBS2</i>   |
| MAP Kinase Kinase Kinase   | <i>BCK1, STE11</i>              |
| MAP Kinases (ERK1, ERK2)   | <i>FUS3, KSS1, MPK1 (SLT2),</i> |
| p65PAK Serine/Threonine Kinase (binds Rac and CDC42)   | <i>STE20</i>                    |
| Protein Kinase C Inhibitor   | <i>BMH1</i>                     |
| Protein Kinase C $\beta$ 1   | <i>PKC1</i>                     |
| Serine/Threonine Phosphatase (PP2C)  | <i>PTC1</i>                     |
| Serine/Threonine Phosphatase 2A (B-subunit)  | <i>CDC55</i>                    |
| Tyrosine Phosphatase   | <i>PTP1, PTP2</i>               |
| Casein Kinase 1  | <i>HRR25, CK11, CK12, CK13</i>  |
| Glycogen Synthesis Kinase 3 $\beta$  | <i>RIM11</i>                    |

## 2.1. General Strategy

In yeast the heptahelical GPCR, Ste2, is engaged in intracellular signal transduction initiated by the  $\alpha$ -factor pheromone (Figure 3) [12, 13]. Compatible with the evolutionarily conserved signaling function of Ste2, its expression in human HEK293 cells led to stimulation of the MAPK Erk1/2 upon incubation with  $\alpha$ -factor [14]. Stimulation of Ste2 in yeast led to its endocytosis, ubiquitination and finally degradation, whereas in HEK293 cells, the  $\alpha$ -factor-induced internalization of Ste2 was not accompanied by significant downregulation of the cellular amount of Ste2. The lack of expression of typical receptor tyrosine kinases, such as the insulin receptor and IGF-1 receptor, in yeast makes Ste2 a perfect target for the identification of the protein motifs which enable the regulation of GPCRs through mammalian tyrosine kinases and vice versa for the impact of GPCR expression and activation on receptor tyrosine kinase signaling, such as desensitization of the

insulin receptor as the molecular mechanism for insulin resistance and T2D. For instance, in the basal state a Ste2-GFP fusion protein becomes targeted to the plasma membranes, but does not undergo endocytosis upon challenge of the yeast cells with insulin, as is typical for the GPCR  $\beta_2$ -adrenergic receptor in course of agonist binding [14]. The failure of insulin to control the intracellular trafficking of Ste2 enabled the construction of a model system consisting of yeast Ste2 and mammalian cells for studying the protein motifs which are responsible for the “linear” or “single-hit” GPCR receptor biology (i.e. agonist binding > receptor activation > endocytosis > ubiquitination > proteasomal degradation) induced by typical GPCR ligands, such as  $\beta$ -adrenergic agonists. In higher eukaryotic cells, such as insulin target cells (liver, muscle, adipose), the resensitization and recycling of endocytosed GPCRs is typical, which raises the question about the molecular mechanisms controlling the recycling efficacy and thereby the periods of cell-surface retention. Yeast GPCRs are known to operate independent of cross-talk from/to receptor tyrosine kinases, which could influence the cell surface expression of GPCRs in mammalian cells. However, in yeast the endocytosis of GPCRs is not coupled to their rapid degradation or recycling back to the cell surface. The molecular mode of the control of GPCR trafficking by receptor tyrosine kinases, such as the insulin receptor, turned out to encompass the direct tyrosine phosphorylation of the GPCR as well as the serine/threonine phosphorylation by downstream protein kinases, such as PKB/Akt. Thus the identification of the complete panel of amino acid motifs, phosphorylation sites and downstream binding events with adaptor proteins, such as Grb2, can be achieved by the recombinant introduction of selected regions of the mammalian GPCR of interest into yeast Ste2 and subsequent assaying for the fate of this chimeric receptor in response to the corresponding agonist in animal cells. Thereby it was previously shown that the downregulation by degradation of a chimeric yeast Ste2 upon challenge with insulin is conferred by substitution of the endogenous cytoplasmic domain of Ste2 with that of the  $\beta_2$ -adrenergic receptor [14]. This model system will enable the screening for protein kinases, protein phosphatases and adaptor molecules which are engaged and required in the recycling/resensitization of a mammalian GPCR, such as  $\beta_2$ -adrenergic receptor, under the control of a mammalian tyrosine kinase, such as the insulin receptor, and vice versa, of a tyrosine kinase under the control of a GPCR. Thus, the investigation of such endocytosed Ste2 chimera may be useful for the elucidation of novel drug targets as well as insulin-mimetic and insulin-sensitizing agents for the therapy of diabetes.

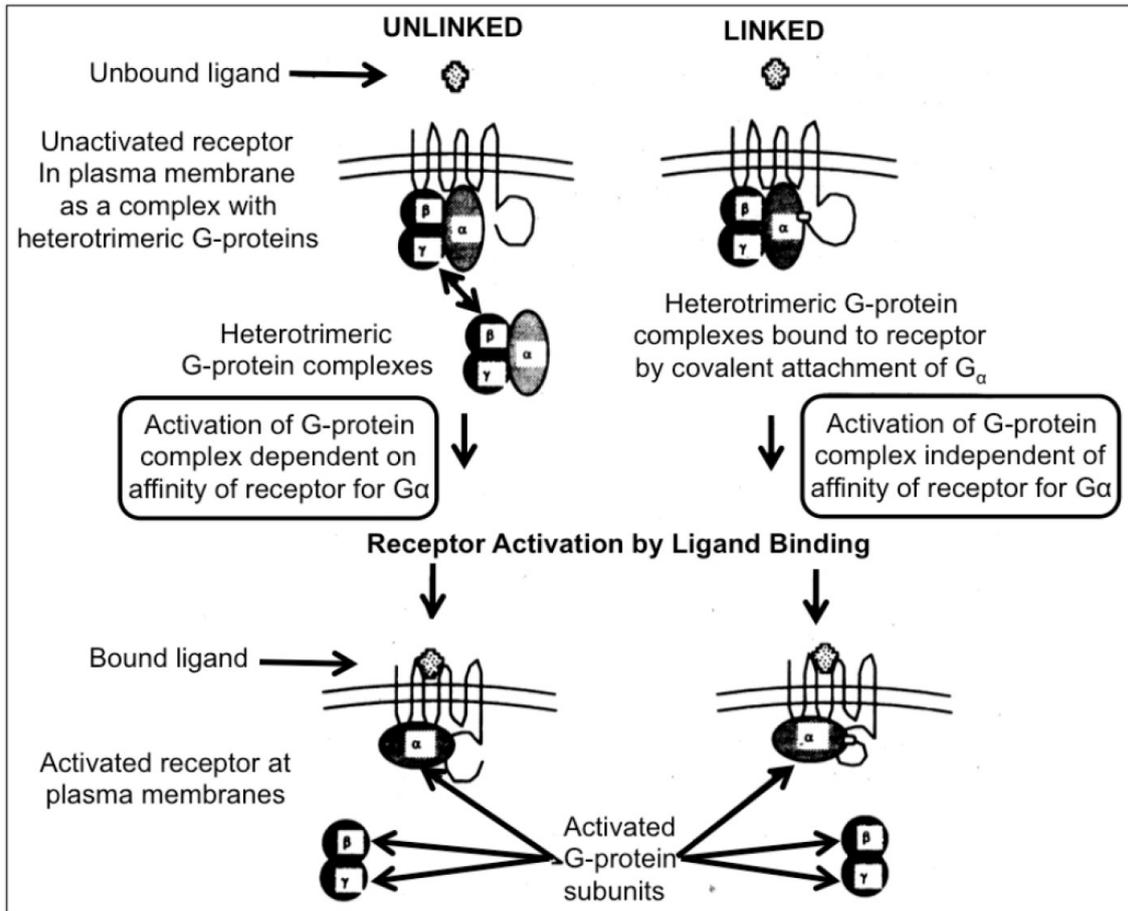
## 2.2. GLP-1 Receptor as Target

The finding that a more pronounced insulin secretion results from the administration of glucose *via* the oral compared to the intravenous route prompted the postulation of the action of so-called incretins [15]. GLP-1 is a members of the incretins which is secreted by intestinal cells in response to nutrient ingestion. GLP-1 contributes to the regulation of blood glucose predominantly *via* the induction of insulin release from pancreatic  $\beta$ -cells [16]. GLP-1 triggers this insulin-releasing effect by binding to and activation of a typical GPCR, the GLP-1 receptor [17]. Thereby, the GLP-1 receptor plays an important role in glucose-dependent insulin release and has gained major interest as a target for the identification of novel (insulin-

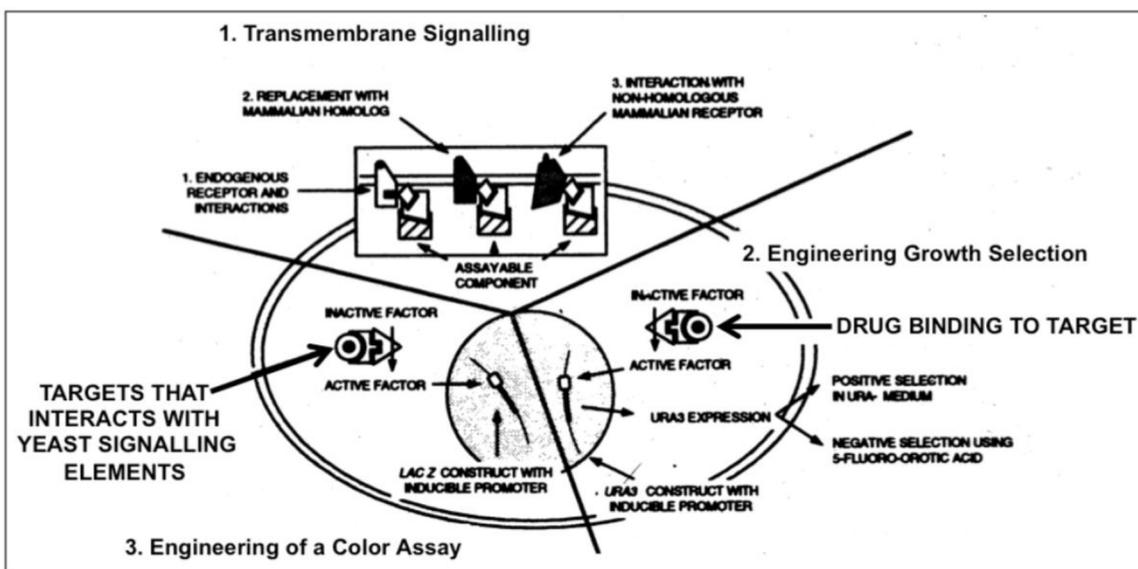
releasing) drugs for the therapy of T2D [18]. Interestingly, T2D is not only characterized by reduced insulin sensitivity and capacity of insulin secretion, but by decreased serum concentrations of GLP-1, in addition [19]. Strikingly, the intravenous injection of GLP-1 peptide causes potent upregulation of glucose-dependent insulin secretion and counteracts hyperglycemia in T2D patients [20]. Unfortunately, the plasma half-life of GLP-1 (7–36 amide) as the active version of GLP-1 is very short due to rapid cleavage to the inactive version GLP-1 (9–36 amide) by the serine protease dipeptidyl peptidase IV [21]. To overcome this limitation and to improve plasma half-life, a number of DPP-IV-resistant peptidic GLP-1 analogues, such as exenatide and liraglutide (Table 4), were developed which meanwhile have been introduced into the therapy of T2D [22].

**Table 3.** Some selected yeast homologs of mammalian cAMP and G-protein signaling components, acylation factors, and lipid kinases with established function in signal transduction.

| Signal Transduction Component                               | Yeast Homolog                        |
|---|--------------------------------------|
| Adenylyl Cyclase-associated Protein (CAP)                   | <i>CAP</i>                           |
| C-Farnesyl-Cysteine Methyltransferase                       | <i>STE14</i>                         |
| Calnexin, Calreticulin                                      | <i>CNE1</i>                          |
| cAMP-specific Phosphodiesterase (type 3)                    | <i>PDE1, PDE2 (SRA5)</i>             |
| Farnesyltransferase, CAAX-specific, Peptide-binding Subunit | <i>DPRI (RAM1)</i>                   |
| GDP-GTP Exchange Factor                                     | <i>BUD5, SCD25</i>                   |
| GDP-GTP Exchange Factor (m-SOS, Ras-specific)               | <i>CDC25</i>                         |
| GDP-GTP Exchange Factor (Rab-specific)                      | <i>DSS4</i>                          |
| Geranylgeranyl Transferase (Rab-specific) $\alpha$ -subunit | <i>MAD2</i>                          |
| Geranylgeranyl Transferase (Rab-specific) $\beta$ -subunit  | <i>BET2</i>                          |
| GTP-Binding Protein (p21Ras)                                | <i>RAS1, RAS2</i>                    |
| GTP-Binding Protein ( $G_{ss}$ )                            | <i>CDC70 (SCG1, GPAL, DAC1)</i>      |
| GTP-Binding Protein ( $G_{b1}, G_{b2}$ )                    | <i>STE4</i>                          |
| GTP-Binding Protein ( $G_{gt}$ )                            | <i>STE18</i>                         |
| GTP-Binding Protein, 70% identical to Rac ( $G_p$ )         | <i>CDC42</i>                         |
| GTP-Binding Protein, Ras-related (Rab1)                     | <i>YPT1</i>                          |
| GTP-Binding Protein, Ras-related (Rab3)                     | <i>SEC4, YPT1</i>                    |
| GTP-Binding Protein, Ras-related (Rab7)                     | <i>YPT7</i>                          |
| GTP-Binding Protein, Ras-related (Rho)                      | <i>Bud1 (RSR1), RHO1, RHO2, RCN1</i> |
| GTPase Activating Protein (Rab6-specific)                   | <i>GYP6</i>                          |
| GTPase Activating Protein NF1 (Neurofibromatosis 1)         | <i>IRAI, IRA2</i>                    |
| Phosphatidylinositol 3-Kinase                               | <i>DRR1, TOR3, VPS34</i>             |
| PI-specific Phospholipase C                                 | <i>PLC1</i>                          |
| Ras-associated GTPase Activating Protein                    | <i>CLA2, (BUD2, ERC25)</i>           |
| Vav (hematopoietic-specific GDP-GTP exchange factor)        | <i>CDC24</i>                         |



**Figure 1.** Two different modes of activation of heterotrimeric G-proteins by receptors are feasible in yeast cells. In the unlinked physiological mode of interaction between receptor and G-protein, the activation depends on the affinity between both partners (left section). A possible “mismatch” between a mammalian receptor expressed in yeast and the endogenous G-protein may be overruled by covalent linkage to G $\alpha$  to the receptor. Nevertheless, the engineered covalent hybrid receptor-G-protein signaling complex in yeast is responsive towards activation/dissociation of the endogenous G $\beta$ /G $\gamma$ -subunits by ligand binding to the mammalian receptor “subdomain” (right section).



**Figure 2.** Engineering yeast for drug discovery requires functional reconstitution of transmembrane signalling (1) which can be coupled to a signaling pathway regulating growth (2) or transcription of a reporter gene (e.g.  $\beta$ -galactosidase) enabling a simple color assay (3).

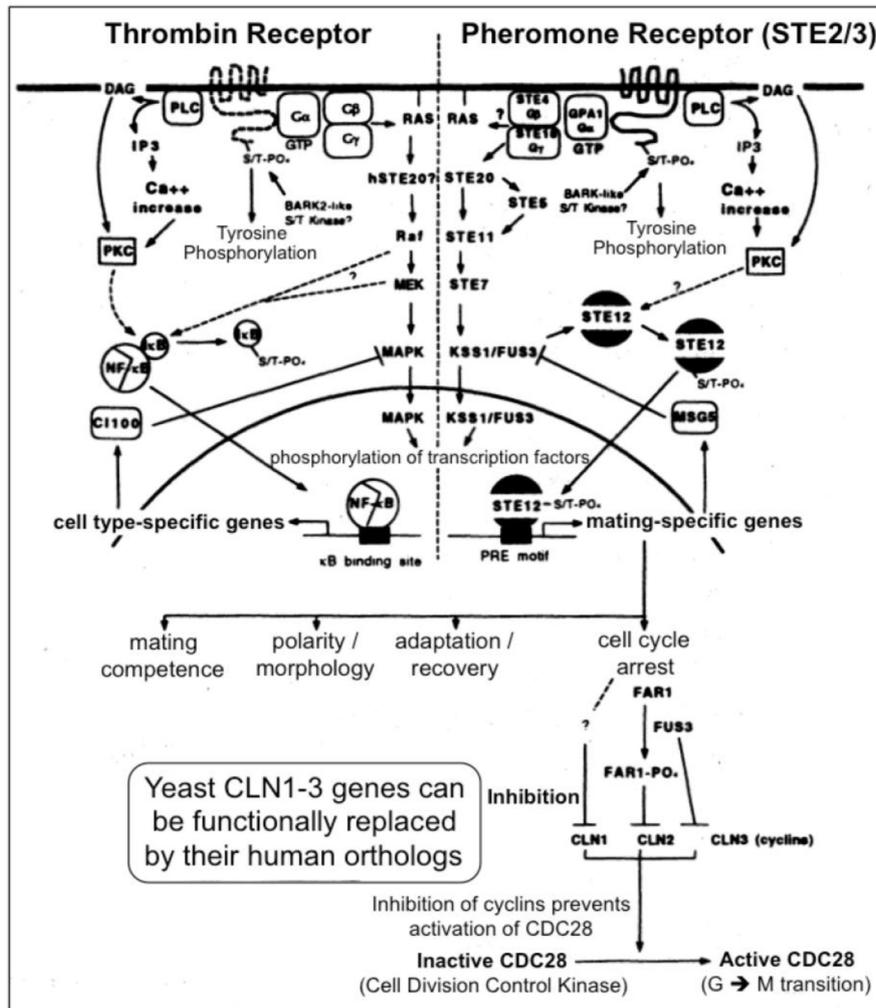


Figure 3. A multitude of orthologous components is engaged in signaling via heterotrimeric G-proteins in yeast (right section) and mammals (left section)

Table 4. Comparison of GLP-1 receptor peptide ligands and relative bias factors (adapted from and modified according to Weston 2014). The sequences of the various peptide ligands for the GLP-1 receptor are aligned to the natural agonist (GLP-1). Deviations in amino acid sequence from GLP-1 of the other GPCR ligands are highlighted in blue. The relative (to GLP-1) bias factor was quantitatively evaluated for each ligand as the change in  $\log(\tau/K_d)$  ratio where a negative value indicates preference for the inhibitory  $G_{i2}$  chimera. Statistical significance was determined using one-way ANOVA with Bonferroni's post-test with each data set compared with GLP-1 (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Data are mean of 5–8 independent experiments + SEM.

| Ligand        | Sequence                                  | Bias           |
|---------------|---|----------------|
| GLP-1         | HAEGTFTSDVSSYLEGQAAKEF I AWLVKGR          | 0.0 + 0.01     |
| Glucagon      | HSQGTFTSDYSKYLDSSRAQDFVQWLMNT             | -1.0 + 0.1***  |
| Oxyntomodulin | HSQGTFTSDYSKYLDSSRAQDFVQWLMNTKRKNKNNIA    | -0.7 + 0.03**  |
| Exenatide     | HGEGTFTSDLSKQMEEEAVRLF I E TLKNGPSSGAPPPS | -0.72 + 0.1*** |
| Liraglutide   | HAEGTFTSDVSSYLEGQAAKEF I A WLVRGR         | 0.0 + 0.001    |

In fact, these GLP-1 mimetics turned out to have important advantages in the control of T2D, such as rapid weight loss and low risk for hypoglycemic episodes, but their chronic application has been implicated with elevated risk of cancer and pancreatitis [23]. Unexpectedly, different GLP-1 mimetics sharing pronounced sequence homology exhibit considerable differences in their clinical profile [24]. This heterogeneity may be explained by the engagement of multiple

isoforms of the GLP-1 receptor or, alternatively, the differential activation of the GLP-1 receptor with regard to its ability to couple to and activate distinct G-proteins and thereby to induce distinct downstream signaling pathways. Thus, much remains to be learnt about the biology of the GLP-1 receptor, in particular the (different) mode(s) of interaction of ligands and the resulting differential consequence(s) for the downstream responses. This inadequate and

incomplete understanding of the molecular mechanisms of GLP-1 action in combination with the concerns regarding the safety of GLP-1 mimetics led to their categorization as “tier 2” of the consensus algorithm for initiation and adjustment therapy for the American Diabetes Association and European Association for the Study of Diabetes. Under this category the “less well-validated” treatments are encompassed and recommended as the last-line treatment of patients. This judgement can only be revised on the basis of a better knowledge of the differential activation and downstream signaling of the GLP-1 receptor in course of ligand binding. Corresponding studies should facilitate the development of GLP-1 mimetic drugs with improved efficacy and safety.

The conventional assays used so far for studying GLP-1 receptor signaling are affected by other signaling pathways which manage to cross-talk to the receptor. Recently a robust and simple yeast-based assay system has been introduced which enables the monitoring of single GPCR-G-protein couplings with the aim to decipher the impact of a defined G-protein subunit on GLP-1 receptor downstream signaling [26]. Previously established test systems for the measurement of the dissociation constants of agonists and antagonists as well as relative binding efficacy of agonists have demonstrated the robustness of signaling assays using *Saccharomyces cerevisiae* cells [27]. Even more importantly, since the GPCR-G-protein interaction is defined in unequivocal fashion, the putative bias of a given (expressed) GPCR or G-protein for the interaction with a certain G-protein or GPCR, respectively, and corresponding consequences for downstream signaling can be elucidated rather conveniently without confounding effects potentially caused by other GPCRs and competing G-proteins. Subsequently, this yeast signaling assay has been adapted to establish the G-protein bias profiles for a number of GLP-1 mimetics (Table 4) [28].

The use of yeast compared to other (e.g. mammalian) test systems has the benefit of providing a relatively zero background for G-protein activation which allows the identification of activation profiles at the individual level. This is of particular importance for the GLP-1 receptor since it is known that this receptor like other GPCRs is known to couple to a multitude of different G-proteins. The predominant one is the  $G_{\alpha_s}$  subunit which upon activation signals for the stimulation of cAMP generation. A minor one is the pertussis toxin-sensitive inhibitory  $G_{\alpha_i}$  [29, 30], the functional outcome of this coupling however remains to be characterized in greater detail [31]. In fact, the productive coupling of GLP-1 to  $G_{\alpha_i}$  was confirmed [28] with the use of the yeast chimeric  $G_{\alpha_q}$  system [26, 32, 33]. GLP-1 as the natural peptide ligand elicited the concentration-dependent induction of the GPA1/ $G_{\alpha_i}$  chimera with significantly diminished potency ( $EC_{50}$ ) and efficacy ( $\log \tau$ ) in comparison to signaling through GPA1/ $G_{\alpha_s}$ . Interestingly, antagonism of the signaling event was left unaltered. These findings can be interpreted with the G-protein subunit expressed being irrelevant for the antagonist affinity and the measured alterations in agonist affinity being due to the preference for a certain G-protein, but independent of the use of the yeast assay system. Significant differences between the two systems were not measured with regard to the dissociation constant for the receptor antagonist, exendin-3. In contrast, for the therapeutically relevant GLP-1 ligands, liraglutide and exenatide, considerable but previously unrecognized differences in G-protein

signaling were detected with exenatide exhibiting a pronounced bias for the  $G_{\alpha_i}$  pathway. Subsequently, this approach was extended to the investigation of small-molecule allosteric compounds and the closely related GPCR glucagon receptor. In conclusion, the yeast GPCR assay system allows the reliable and system-independent measurement of the pharmacological properties of putative GPCR-based drugs. The glucagon receptor as well as the GLP-1 receptor belong to the secretin (family B, class 2) GPCR family with 15 members. The peptidic ligands for those receptors typically consist of 27 to 84 amino acids.

### 2.3. The Use of Permeable Yeast Cells

For decades the investigation of GPCRs in yeast was not favored by the researchers since the yeast cell wall was thought to prevent their naturally occurring peptidic ligands from reaching the (periplasmic face of the outer) plasma membranes, where the ligand binding domain of the (ectopically) expressed GPCRs will be located. However, recently the ectopic expression of two corticotropin-releasing factor receptor subtypes [34, 35] as well as the functional co-expression of the calcitonin receptor-like receptor together with a variety of distinct receptor activity-modifying proteins (RAMPs) [36] has been demonstrated. In apparent conflict with the above assumption of the non-permeation of GPCR ligands across the yeast cell wall, in those cases as is true for the glucagon receptor and the GLP-1 receptor, the yeast-based assay system succeeded in the accurate reproduction of the *in vitro* pharmacology (e.g. relative binding affinities, G-protein coupling), albeit with absolute binding affinities typically below those measured in corresponding mammalian test systems. Nevertheless or to overcome the latter limitation permeable yeast cells have been developed.

Microbial cells have evolved an impermeable cell wall and plasma membranes that allow them to survive in the environment. Therefore the drug target may not easily accessible to compounds in high-throughput screens that use microbial cells, which in consequence are likely to miss those compounds being unable to penetrate across the cell wall and plasma membrane barriers. In addition, many microbes have very effective efflux systems that pump out compounds. These efflux systems are similar to the multidrug resistance (MDR) transporters found in tumor cells. One of the large classes of efflux systems, or transporter, is called the ATP-binding cassette transporters or ABC transporters. The ABC transporters are conserved from bacteria to man [37]. With such drawbacks, can microbial- and yeast-based screening be effectively used for drug discovery? Genetic and molecular technology has made it possible to remove some of these barriers and make screen development and screening in unicellular lower eukaryotes a viable, inexpensive, and productive alternative to other screening systems. Among lower eukaryotes, *Saccharomyces cerevisiae* have been the most popular because of the genetic manipulations feasible with this organism. Since it is an eukaryote, it is often considered to be a more realistic system for screening for mammalian drug targets as compared to prokaryotes, such as *E. coli*. However, yeast is slower to grow than *E. coli*, taking 48 h to grow to adequate cell densities, while *E. coli* can be used within 6 to 8 h of growth. The genetic manipulations in *E. coli* are considerably less difficult than with yeast and *E. coli* are more permeable than yeast.

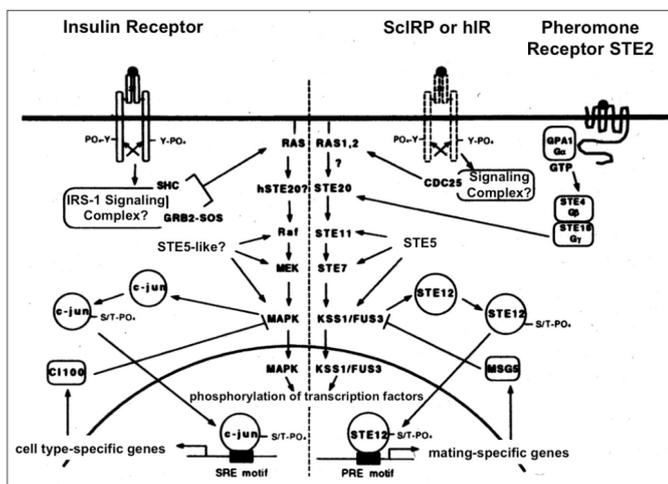
In fact, wild-type *Saccharomyces cerevisiae* is quite impermeable owing to their cell wall and plasma membranes. The cell wall is considered to be latticelike and allows most small molecules to permeate through. However, the plasma membranes are considered to be quite impermeable. Strikingly, previously it has been noted that yeast cells are actually permeable and the lack of drug effect is the result of the activity of multiple efflux systems, belonging to the family of ATP-binding cassette transporters (MDR), called PDR, that rapidly pump out compounds. The transcription factors, *pdr1p* and *pdr3p*, down-regulate the expression of hexose transporters, HXT11 and HXT9, which in turn up-regulate the expression of PDR (Figure 4). Thus deleting the hexose transporters, HXT11 or HXT9, confers pleiotropic drug resistance on yeast while overexpression of these transporters results in increased sensitivity to drugs. Furthermore, deletion of the regulators of the promoter for the ATP-binding transporters, PDR1 and PDR3, in HXT11 and HXT9 over-expressing strains, results in supersensitive yeast [38]. These mutant strains are ideal organisms for use as host strains for the development of screens. Improved cell permeability was also reported for the yeast strains with deletion of the *YOR1* gene, which encodes another ATP-binding cassette transport protein [39]. In addition to transporter mutants, mutants in the ergosterol pathway, such as in *ERG6*, are also more permeable to small molecules used in screening compared to wild-type strains [40]. However, the mutants in the ergosterol pathway have the disadvantage that their growth is negatively affected, and they are difficult to transform.

simplicity and convenience of the ectopic expression of GPCRs in yeast would be compatible with the introduction of patient-specific mutations into GPCRs with the aim to study and to quantitatively analyze their impact on the ligand-induced signaling capability of the receptor and possibly to efficiently screen for ligand mimetics, which bypass the defect. Thereby, the yeast-based assay system could contribute to the development of personalized drugs.

### 3. Insulin Receptor and Signaling as Target

This approach originated during an attempt to study the molecular basis for tyrosine kinase-linked signal transduction in a cell system in which there is no detectable endogenous receptor tyrosine kinase-coupled regulatory processes, namely, the fission yeast *Schizosaccharomyces pombe*. Expression of selected mammalian signaling elements in such a naive system might support productive interactions between heterologous components while eliminating involvement and cross-talk with host cell tyrosine kinase-associated control systems. *S. pombe* has been used by others to study the function and interactions of selected mammalian signaling elements, including PKC isoforms, Src, Csk, Raf-1, Ras, PI-3K and MAPKK. Furthermore, high level expression of the platelet-derived growth factor receptor and its substrate phospholipase C $\gamma$  (PLC $\gamma$ ) in *S. pombe* led to autophosphorylation and substrate phosphorylation at tyrosine residues. Therefore, appropriate cleavage and oligomerization of the expressed human receptor seem to be possible. Potential assays may rely on (i) modulation of growth, (ii) insulin binding, (iii) autophosphorylation of the receptor at tyrosine residues, (iv) tyrosine phosphorylation of heterogeneously expressed authentic or engineered substrates, such as IRS-1 and PI3K as well as fusion proteins or fragments derived thereof, or artificial substrates, e.g. EEEY and (v) phosphorylation of yeast substrates (Figure 4). These experimental designs should demonstrate, that heterologous expression of selected signaling elements in yeast will permit the detection and analysis of functional insulin receptor activation and pave the way for further systematic and detailed molecular dissection of a wide range of interactions, central to insulin receptor kinase-coupled signal transduction processes as well as for the identification of peptidic or small peptidomimetic molecules, which manage to strengthen or disrupt those interactions.

Alternatively, the assays for human receptor activation may be based on signal transduction pathways activated by receptor tyrosine kinase activity: I. The soluble domain of the human insulin receptor can be stably expressed at the plasma membranes of *Saccharomyces cerevisiae* [42]. II. Tyrosine phosphorylation of some yeast proteins is increased upon incubation of intact cells or spheroblasts with human insulin [43]. This raises the possibility that the tyrosine kinase activity of the human insulin receptor may initiate signal transduction cascades leading to phosphorylation of endogenous substrates, and their human orthologs expressed in yeast. This will require (i) cloning of the yeast signaling proteins that respond to the human insulin and insulin-dependent (tyrosine/serine/threonine) phosphorylation, (ii) identification and (iii) expression of their human orthologs. III. Low levels of expression of the insulin receptor kinase domain may lead to a very modest but reproducible slowing of growth, high expression



**Figure 4.** Insulin signal transduction may regulate the MAPK cascade in yeast (right section). Insulin signaling in insulin-responsive mammalian cells via the homologous Ras-MAPK pathway is shown for comparison (left section). In yeast coupling of the endogenous (ScIRP) or heterologously expressed human (hIR) insulin receptor to the MAPK pathway could occur via the heterotrimeric G-protein, GPA1/STE4/STE18 to STE20 (which is also used by the pheromone receptor during the mating response) or via CDC25 to Ras1/2, respectively, which may be analogous to mammalian cells where the insulin receptor transduces its signal to Ras via the GDP-GTP exchange factor SOS.

It would be useful and feasible to extend this technology for the inclusion of many GPCRs or other components, which putatively interact with the receptor *in situ* (e.g. regulatory proteins of G-protein signaling, RAMPs), in order to recognize any (positive or negative) effect of an interaction built up on the receptor pharmacology and the resulting (patho)physiological changes. Moreover, the relative

may result in stronger inhibition as has been reported previously for overexpression of the constitutively active human insulin receptor in cultured CHO cells [44]. In general, clear-cut effects of activated yeast or human receptors on downstream signaling proteins of yeast or human origin represent potential parameters of measurement for a yeast-based screening assay. Taken together, yeast and other lower eukaryotic unicellular organisms have already proven their usefulness in various aspects of pharmaceutical development and, no doubt, their impact will further increase during the next years.

#### 4. Endoplasmic Reticulum Stress and Pancreatic $\beta$ -Cell Dysfunction as Target

Failure of insulin secretion caused by reduction in the number and functionality of pancreatic  $\beta$ -cells has been considered as the hallmark of T2D for decades [45, 46]. The majority of T2D patients is characterized by overweight or obesity which in general is correlated to elevated levels of plasma free fatty acid (FFA) levels [47, 48]. Albeit being still a matter of intense debate, this hyperlipidemic state could be (causally) involved in the loss of functional  $\beta$ -cells through a pathophysiological process called lipotoxicity [49]. Importantly, the operation of lipotoxic processes under conditions of overfeeding and excessive calorie uptake, as often prevalent during obesity and T2D, has also been reported for a number of non- $\beta$ -cells, such as adipocytes, myocytes, hepatocytes and brain cells, and linked to their desensitization towards insulin challenge [50–53]. The molecular mechanisms underlying the FFA-induced lipotoxicity have been investigated *in vitro* during the past two decades in course of direct incubation of cultured  $\beta$ -cells, pancreatic islets or insulin target cells and, more recently, of yeast cells (*Saccharomyces cerevisiae*) [54–56]. Strikingly, despite the tremendous differences between these cell types considering their morphology, structure and physiology, they apparently display pronounced similarity with regard to their susceptibility and viability towards FFA (Table 5). This argues for

conservation, at least in part, of lipotoxic mechanisms along the evolution of eukaryotes from yeast to humans. Importantly, in  $\beta$ -cells, insulin target cells and yeast cells the extent of the lipotoxic effects provoked by the FFA critically depends on the number of their carbon atoms and the degree of their saturation. The data available so far are consistent with saturated (SFA) and long-chain FFA (e.g. palmitate C16:0, stearate C18:0) exerting the most adverse effects, whereas saturated and short(er)-chain ones (e.g. myristate C14:0 and below) as well as long-chain unsaturated fatty acids (UFA) equipped with one to several double bonds (e.g. palmitoleate C16:1, oleate C18:1, linoleate C18:2 and linolenate C18:3) exhibiting considerably lower harmfulness [57–59]. In addition, the concentration-dependent abrogation of the SFA-triggered lipotoxic effects, in general, and of cell death, in particular, by excess of UFA has meanwhile been amply documented [60, 61].

Most importantly, in both  $\beta$ -cells and yeast cells the SFA-triggered lipotoxicity leads to the initiation of the so-called stress response in the endoplasmic reticulum (ER) or unfolded protein response (UPR)(Table 5). The ER represents the site for a number of essential physiological processes, such as  $\text{Ca}^{2+}$ -homeostasis, (phospho)lipid biosynthesis and biogenesis of proteins, which have to be transported to intracellular organelles and the plasma membranes or secreted into the extracellular milieu. Prior to guidance into the secretory pathway the membrane and secretory protein precursors have to be correctly folded and assembled in the ER. Any imbalance between the folding capacity of the ER and its passenger protein load will ultimately cause ER stress [62]. ER stress has to be counterbalanced by the multiple strategies of the UPR, such as proteolytic degradation [63] to preserve or re-gain the structural and functional integrity of the ER despite the accumulation of misfolded polypeptides [64, 65]. It is believed that the SFA-triggered cell death is mediated through ER stress and the failure to cope with it in the presence of high concentrations of SFA, which finally will result in apoptosis.

**Table 5.** The effects of exposure of yeast- and  $\beta$ -cells towards palmitate in comparison.

|                | UPR pathway |      |      | Apoptosis/<br>Growth arrest | Misfolded<br>proteins | Depleted ER<br>$\text{Ca}^{2+}$ stores | Altered ER morphology |
|----------------|-------------|------|------|-----------------------------|-----------------------|--|-----------------------|
|                | Ire1        | PERK | ATF6 |                             |                       |  |                       |
| $\beta$ -cells | +           | +    | +(?) | +                           | +                     | +                                      | +                     |
| yeast          | +           | None | None | +                           | +                     | not tested                             | +                     |

In higher eukaryotes, a critical pathway engaged by the UPR, which senses for unfolded membrane protein precursors, is constituted by the inositol-requiring enzyme 1 (IRE1) cascade [65], which in  $\beta$ -cells seems to be upregulated in response to long-chain SFA, such as palmitate. The IRE1 cascade constitutes the only UPR pathway which is conserved from yeast to humans. In *Saccharomyces cerevisiae* the accumulation of misfolded proteins triggers dimer formation of Ire1p which results in upregulation of its endogenous endoribonuclease activity. In non-stressed yeast and  $\beta$ -cells the ER-resident protein of the HSP70 family, Kar1p and BiP, respectively, binds to Ire1(p) for suppression of its activation. In stressed cells harboring excess of misfolded proteins in the ER Kar1p / BiP manages

to dissociate from Ire1(p) and thereby enables Ire1(p) dimerization which finally leads to its autophosphorylation and activation [64]. The Ire1p endoribonuclease activity is directed against the mRNA of the transcription factor Hac1p which interacts with the promoter of UPR elements (UPREs) and controls the expression of about 5% of the genes in yeast (and is the yeast ortholog of mammalian XBP1). Importantly, in yeast upregulation of the Ire1p cascade in the presence of high concentrations of SFA was observed using a reporter gene (*LACZ*) assay which monitored the transcription activity of four UPRES, i.e. the amounts of active Hac1p/XBP1, as reflected in the measured  $\beta$ -galactosidase activity [66]. High intracellular concentrations of SFA in yeast as well as in  $\beta$ -cells, which may result either from blockade

of endogenous fatty acid desaturation or from the presence of palmitate in the incubation medium, have been reported to provoke full induction of the Ire1(p) cascade. Consistent with the beneficial effects of UFA, Ire1(p) activation was diminished in concentration-dependent fashion in the presence of oleate [67].

## 5. Adipocyte Lipolysis as Target

The concentration of FFA in the blood of mammals which are tightly linked to their metabolic disease state, such as T2D in case of chronically elevated levels, critically depends on the efficacy of lipolysis in the various adipose tissue depots [68]. Insulin with its primarily anabolic function in the body manages to restrict lipolysis and to foster the incorporation of FFA, which are ultimately derived from the ingested nutrient lipids, into neutral triacylglycerol, which becomes stored in adipocytes, during the postprandial state. Missing suppression of adipocyte lipolysis by insulin has been considered since decades as the major physiological defect which is causative for metabolic diseases, such as T2D and obesity, through the induction of insulin resistance in peripheral tissues and dysfunction and death of  $\beta$ -cells through the operation of lipotoxic mechanisms (see above) [69–71].

Triacylglycerol is lipolytically degraded to FFA and glycerol through the joint action of tri-, di- and monoacylglycerol lipases [72–75] with the most recently unraveled Adipose Triglyceride Lipase (ATGL) [76–78] exerting the major portion of the triacylglycerol hydrolase activity in adipose, muscle and liver tissues and representing the rate-limiting enzyme for lipolysis. This is manifested in the observation that up- and downregulation of ATGL expression unequivocally leads to increase and decrease, respectively, of both the basal and the cAMP-induced triacylglycerol hydrolysis [79–81]. It has been accepted for decades that the rate of lipolysis is primarily under the short-term and post-translational control of the cAMP-dependent signaling pathway through phosphorylation of the lipid droplet coat protein perilipin and the diacylglycerol lipase HSL by PKA leading to rapid stimulation of lipolysis, whereas insulin-induced blockade of cAMP-dependent signaling *via* Akt-dependent [82] and -independent [83] mechanisms causes inhibition of lipolysis.

In addition, insulin and other physiological anti-lipolytic stimuli, such as feeding and  $\alpha$ -adrenergic hormones, have to elicit a long-term inhibitory effect on lipolysis which is based on the suppression of its rate-limiting enzyme, ATGL, most likely as a consequence of downregulation of ATGL expression. Recently, a novel molecular mechanism for the negative regulation of ATGL expression by insulin and nutrients has been identified, the mTORC1-dependent pathway which blocks lipolysis by reduction of ATGL transcription [84]. Interestingly, in *Drosophila* a similar mechanistic link between dTORC1 and the ATGL homologue Brummer lipase [79, 85] was demonstrated recently [86, 87], which even can be extended to the insulin receptor [88]. The apparent evolutionary conservation of this anti-lipolytic molecular mechanism strongly argues for its essential physiological role in eukaryotes, in general, and in humans, in particular, as well as makes it to an attractive source for targets for the therapy of metabolic diseases.

For elucidation of the molecular mode of mTORC1 action, yeast as a model organism was used based on the knowledge, that *Saccharomyces cerevisiae* harbors a functional ortholog of ATGL, the triglyceride lipase Tgl4p (Table 1) [80]. The putative involvement of the Tor1-dependent pathway in the expression of Tgl4 was studied by growing *S. cerevisiae* in the presence of the specific Tor1/mTORC1 inhibitor, rapamycin [81]. In fact, rapamycin led to a considerable and specific upregulation of the Tgl4p mRNA expression. For identification of the transcription factors engaged in Tgl4 expression, a *S. cerevisiae* deletion library was screened [81]. Interestingly, yeast cells lacking Msn4 were found to have increased basal levels of Tgl4 mRNA and to be almost insensitive towards the stimulatory effect of rapamycin on Tgl4 expression. The residual and very moderate positive effect of rapamycin on Tgl4 transcription in Msn4-defective yeast cells argues for the participation of factors other than Msn4 in the transcriptional control of Tgl4 expression by TOR1. Strikingly, orthologs of yeast Msn4p in mammals were reported to constitute a family of early growth response transcription factors, among them the well-known members Egr1 (Krox24), which are known to play important roles in adipocyte differentiation [89, 90] and regulation of cholesterol biosynthetic gene expression [91–93], and Egrs (Krox20) [94]. These data strongly suggest that the regulation of ATGL transcription by the mTORC1-Egr1 pathway is important for the control of lipid metabolism. Otherwise it would not have been conserved along evolution from yeast *via Drosophila* [86, 87] to mammals [84].

## 6. Use of Yeast for Drug Target Identification and Validation

Although yeast-based systems are less complicated than mammalian cell-based systems, orthologs of many mammalian proteins are found in unicellular lower eukaryotes. In addition, many other mammalian proteins that do not have sequence homology but do have functional homology can be used to complement functions in yeast. Unlike in higher eukaryotes, the functions of about half of the yeast genes are known on the basis of amino acid sequence similarity with other proteins of known function [95, 96]. This is an enormous resource that is being used for functional analysis. History has shown us that biological mechanisms revealed from the study of lower eukaryotic cells will be applicable to higher eukaryotes. In the future, knowledge of the function of yeast proteins will help in elucidating the function of many mammalian proteins. The similarity between living organisms was noted by Jacques Monod when he said “What is true for *Escherichia coli* is true for the elephant, except more so” [97]. In the simplest approach, functional complementation can be used to derive a screen in which the activity of the heterologous gene is essential for survival. This approach has been successful even in cases where protein homology is limited, as long as the relevant biological activity is complementary. Yeast-based systems can also be used to define interactions with other proteins. The more difficult approach is to manipulate the heterologously expressed gene to obtain a surrogate phenotype and create “designer yeast”.

### 6.1. Complementation of Homologous Target Proteins

Many currently used drug targets for the therapy of T2D are GPCRs, such as the glucagon and GLP-1 receptors (see above).

Antagonists have been identified by ligand- displacement assays using mammalian cells or their membrane preparations. Agonists have been generally identified by functional assays. Yeast-based systems have been adapted to identify agonists and antagonists of GPCRs. The mating factor receptor in *S. cerevisiae*, Ste2, is similar in structure to mammalian GPCRs. Mammalian GPCR can be used to replace Ste2, so that the GPCR can signal through the mating factor signaling pathway when activated by the GPCR agonist [98]. In order to get efficient downstream coupling with the mating factor pathway kinases, the amino-terminal domain of the G<sub>α</sub> protein was deleted [99]. The GPCRs expressed in this manner in yeast are useful for screening for agonists. Coupling can also be obtained with the natural yeast G<sub>α</sub> protein [100, 101].

The natural ligands of GPCRs can be large peptide ligands, such as for the GLP-1 receptor (see above). In mammalian cells, these GPCRs can be activated by these peptides as well as their derivatives. Antagonists are identified by finding compounds that can displace these peptides. Short peptides can be coexpressed with GPCRs in yeast to develop functional antagonist screens as well as for identifying ligands for orphan GPCRs [102]. Orphan GPCRs are those receptors that have been cloned by sequence homology to known GPCRs but whose function and natural ligands are not known. Peptide libraries have been expressed with secreted sequence tags that are secreted across the plasma membranes of yeast where they come into contact with the GPCRs. Using these peptide libraries, those peptides that specifically interact with and activate the receptor in an autocrine fashion can be identified.

Potassium channels are also important drug targets for the therapy of T2D, among them the ATP-dependent potassium channel (K<sup>+</sup>-ATP) constituted by pore-forming Kir and ligand-binding SUR subunits [103, 104]. Functional screens for K<sup>+</sup> channel openers and blockers involve expensive equipment and are technically difficult to perform [105]. Therefore simpler assays for developing high-throughput screens have been appreciated. A simple functional screen was developed in *S. cerevisiae* using complementation of the TRK1/2 potassium transporter knockouts [106]. The inwardly rectifying potassium channel IRK1 has also been expressed in yeast to complement the Trk transporter defect. In the strain expressing the IRK1 channel, the ion channel activity correlates well with the growth phenotype and with patch clamp experiments in *Xenopus* oocytes expressing these channels.

The influenza M2 channel has been expressed in *S. cerevisiae* [105]. The influenza M2 channel is a proton channel that is expressed in infected cells. Its function is to increase the acidity of the milieu in which the virus sheds its capsid. When expressed in *S. cerevisiae*, the M2 proton channel increases the permeability of yeast plasma membranes to ions resulting in loss of yeast cell viability. In order to develop a screen to find influenza M2 protein inhibitors, it was expressed from a galactose-inducible promoter. The screen was designed to find compounds that permit growth and rescue the cells from the permeabilizing effects of M2 protein when the growth medium is supplemented with galactose. Channel screens designed in *S. cerevisiae* have been useful for high-throughput screening. However, it has to be considered that yeast is slowly growing, and expression of channels in this microbe is difficult and time-consuming.

## 6.2. Expression of heterologous target proteins

Screens to find ligands for steroid hormone receptors such as retinoid receptors, which constitute an important class of drug targets for T2D and cardiovascular diseases, have been designed in *S. cerevisiae* [107]. Steroid hormone receptors occur intracellularly and are built up from a ligand-binding domain, a dimerization domain, and a transactivation domain. When ligands induce these receptors to homo- or heterodimerize, their transactivation domain binds the specific response elements and activates specific promoters. The dimerization of the steroid hormone receptor followed by binding and transactivation of specific promoters can be studied in yeast. Homodimerization has been demonstrated using retinoic acid receptors, thyroid hormone receptors, and estrogen receptors [108, 109]. Heterodimerization with the RXR retinoid receptor can also be demonstrated [107]. In this system, the RAR retinoid acid receptors respond to a number of retinoids, but RXR responds only to the RXR-specific 9-*cis* isomer of retinoic acid [107]. Because all mammalian cells have many representatives of the steroid hormone receptor family expressed naturally, yeast-based systems offer cells with a “null” background activity for studying specific interactions.

Tyrosine-specific protein kinases and phosphatases are important drug targets for the therapy of T2D. Cell-free assays have been popular for this class of targets since the enzymes are easily produced by recombinant means and tyrosine-specific phosphorylation of the natural or an appropriate artificial substrate protein is simple to detect using labeled ATP. An alternative method using *Schizosaccharomyces pombe* has been published that will find inhibitors that are non-toxic to yeast as well as cell-permeable [110]. The prototypic tyrosine kinase, *Src*, was expressed under the control of the inducible promoter. Induction of *Src* results in cell death, and growth rescue can be used for the identification of inhibitors. To adapt the screen for identifying phosphatase inhibitors, this system was modified by co-expressing tyrosine phosphatase on a second plasmid. When the kinase and phosphatase are coexpressed, the cell survives the detrimental effects of kinase expression. Tyrosine phosphatase inhibitors can be identified in this system by looking for compounds that selectively kill the strain co-expressing the kinase and phosphatase [110].

## 6.3. Detection and Analysis of Protein-Protein Interaction

Proteins carry out their function in most cases by interacting with other proteins. The yeast two-hybrid system, developed by Fields and Song [111], has revolutionized the study of protein-protein interactions. In this system, the transcription factor, GAL4 from *S. cerevisiae*, is used to set up the assay. GAL4 has two domains, a site-specific DNA-binding domain and an acidic region that is required for transcriptional activation. The DNA-binding and activation domains can be coded by separate genes as long as they are brought together in a heterodimer to reconstitute a functional transcription factor. The system is designed so that when GAL4 binds the GAL4-binding domain on the promoter, *LEU2* and/or *HIS3* are expressed. Functionally competent chimeric proteins can be made that consist of the DNA-binding domain fused to one protein of an interacting pair and the activation domain fused to the second protein of the interacting pair. Interaction of the proteins that are constructed as

chimeras of the activating and DNA-binding domains allow the yeast to grow in the absence of histidine and leucine, thus providing a selective advantage. The yeast two-hybrid system is widely used for identifying homo- and heterodimerizing proteins as well as to develop screens to find compounds that can block two proteins from interacting with each other [112].

The yeast two-hybrid system has been modified to measure the dissociation of interacting proteins by using the *URA3* reporter [113]. Yeast cells expressing *URA3* can grow in medium without uracil. When 5-fluoroorotic acid (FOA) is introduced into the medium, *URA3* expressing cells take up FOA and transform it into a toxic compound. Thus the expression of the reporter gene is toxic and provides a powerful selection procedure. This FOA system is used in the “reverse two-hybrid” system, providing a selective growth advantage and a more powerful system for screening. In the “reverse two-hybrid system”, the interacting protein is expressed inducibly, and only when the interacting proteins are blocked do the cells survive. GAL4 and LexA transcription factors are most often used in the yeast two-hybrid system. In two-hybrid screens, it is useful to have two separate reporter constructs to help in sorting “hits”. Reporters such as *Leu2* and *LacZ* can be expressed in the same cell.

The yeast two-hybrid system has been used to develop screens for ligand-receptor interactions, including peptide hormone receptors, such as the GLP-1 receptor, and the receptor tyrosine kinases, such as the insulin receptor [114–116]. Specific and reversible ligand-receptor interactions between growth hormone and growth hormone receptor, VEGF and KDR, can be studied using the yeast two-hybrid system. Ligand-dependent receptor dimerization can also be studied using three expression plasmids in which the receptor is expressed as a fusion protein with both the DNA-binding protein as well as the activation domain. The ligand is expressed from a third plasmid. When the ligand binds the two receptors, the DNA-binding domain and activating domains are pulled together and GAL4 is activated.

The yeast two-hybrid system has been adapted to study protein-protein, protein-RNA, protein-DNA, and protein-small molecule interactions [117]. A one-hybrid system has been developed that utilizes *cis*-acting sequences to identify DNA-binding proteins that can initiate transcription [118]. A yeast three-hybrid was developed to study RNA-protein interactions that are especially useful for developing screens against viruses [119]. In this system, the hybrid RNA containing sites recognized by the RNA-interacting proteins links the two-hybrid proteins containing the DNA-binding and activation domains, respectively. The yeast two-hybrid system has been recently applied to find inhibitors of the N type calcium channel [120, 121]. Alternative screening techniques use mammalian cells to measure calcium channel activity with electrophysiological and spectrophotometric methods to measure calcium influx. These methods are labor intensive, difficult, and not compatible with high-throughput screening. In the yeast two-hybrid system, the interacting, regulatory portion of the  $\alpha 1$  subunit of the channel fused to the Gal4 activation domain and the full length  $\beta 3$  subunit fused to the yeast Gal4 DNA-binding domain were expressed. The system could be adapted to find inhibitors of specific calcium channels by selecting the specific interacting domains.

## 7. Conclusions

Unicellular lower eukaryotes provide an alternate platform for high-throughput screening. Those systems are inexpensive to run and screens can be developed rapidly. Many modular systems are available that are adaptable to important classes of drug targets, such as GPCRs, single-transmembrane receptors such as growth factor tyrosine kinases and phosphatases and ion channels, which are of critical importance for the therapy of metabolic diseases, such as T2D. Functional screens are becoming necessary for developing screens for proteins whose biological functions are not yet known as well as on proteins that interact with new proteins. Because of the ease with which new targets can be explored, functional cell-based screens are becoming the preferred method for finding leads for drug discovery. Test systems based on unicellular lower eukaryotes provide simple and cost-effective means for the identification and validation of drug targets and, most importantly, for the discovery of novel drugs by functional screening.

As thousands of new potential drug targets from genomic information and protein interaction studies have meanwhile been identified, the future of screening is in using chip technology [122, 123]. Systems based on unicellular lower eukaryotes are especially suited for the delivery to microchips. Yeast is robust and is easy to handle and distribute. Thousands of yeast cells can be deposited on chips in a high-density format. In addition, the surface charge on *S. cerevisiae* could be used to array the organisms into the desired format. Currently, the limitation is the sensitivity of reading colorimetric reporters, and alternatives are being investigated. Biosensors and transducers could be used to detect thermal, immunologic, or optical changes [124, 125]. Glucose sensing amperometric systems are being used in clinical microbiology and could be developed for high-throughput screening [126]. Each of the microbial systems described can be adapted to use the reporter that is suitable for the high-density format on chips for screening. Consequently, a yeast genetic system has been designed for the identification of small-molecule inhibitors of protein-protein interaction on the basis of nanodroplets [127].

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