The activation mechanisms of G protein-coupled receptors : the case of the adenosine A2B and HCA2/3 receptors

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Chapter 2

Human G protein-coupled receptor studies in *Saccharomyces cerevisiae*

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Abstract

G protein-coupled receptors (GPCRs) are one of the largest families of membrane proteins, with approximately 800 different GPCRs in the human genome. Signalling via GPCRs regulates many biological processes, such as cell proliferation, differentiation, and development. Moreover, many hormones and neurotransmitters are ligands for these receptors, and hence it is not surprising that many drugs, either mimicking or blocking the action of the bodily substances, have been developed. It is estimated that 30-40% of current drugs on the market target GPCRs. Further identifying and elucidating the functions of GPCRs will provide opportunities for novel drug discovery. The budding yeast *Saccharomyces cerevisiae (S. cerevisiae)* is a very important and useful platform in this respect. There are many advantages of using a yeast assay system, as it is a cheap, safe and stable; it is also convenient for rapid feasibility and optimization studies. Moreover, it offers "null" background when studying human GPCRs. New developments regarding human GPCRs expressed in a yeast platform are providing insight into GPCR activation and signalling, and facilitate agonist and antagonist identification. In this chapter we summarize the latest findings regarding human G protein-coupled receptors in studies using *S. cerevisiae*, ever since the year 2005 when we last published a review on this topic. We describe 11 families of GPCRs in detail, while including the principles and developments of each yeast system applied to these different GPCRs and highlight and generalize the experimental findings of GPCR function in these systems.

Introduction

G protein-coupled receptors (GPCRs) constitute one of the largest families of proteins in the mammalian genome[1, 2]. In the human genome, approximately 800 GPCR sequences have been identified, a relatively large number of which the cognate ligands are still unknown for[3, 4].

GPCRs are activated by a wide variety of extracellular stimuli, such as neurotransmitters, hormones, and growth factors. Upon stimulation, GPCRs exert their role as surface signalling protein by transferring extracellular signals to target molecules in the cytosol or nucleus. Signalling via GPCRs regulates many biological processes, such as cell proliferation, differentiation, and development. They also are central players in (patho)physiologic conditions, such as immunity, blood pressure and kidney function.

GPCR signalling is initiated through receptor activation by an agonist. The conformational change caused by agonist binding allows the GPCR to recruit an associated G protein by acting as a guanine nucleotide exchange factor on the α-subunit of the G protein[5]. Subsequent exchange of GDP for GTP in this subunit causes the other subunits of the G protein, G_{β} and G_{γ}, to dissociate as a $\beta\gamma$-dimer. Both G_{α}-GTP and $G_{\beta\gamma}$ can modulate various cellular signalling pathways, including regulation of adenylate cyclases, phospholipases and the mitogen-activated protein kinase (MAPK) pathway, ultimately leading to expression of target genes[6]. In mammals, several isoforms of each subunit are expressed, many of which have splice variants, resulting in a great amount of combinations of G proteins. Four main classes of these heterotrimeric G proteins are defined based on the sequence and function of their α-subunit: $G_{\alpha s}$, $G_{\alpha q}$, $G_{\alpha i}$, and $G_{\alpha 12}$[6].

The downstream effects of GPCR stimulation depend on the type of GPCR which is activated. Members of the GPCR superfamily share the same basic architecture of 7 transmembrane (7TM) α-helices, an extracellular N-terminal segment and an intracellular C-terminal tail. The 7TM α-helices are connected by three intracellular (IL1, IL2, and IL3) and three extracellular (EL1, EL2, and EL3) loops[7]. Based on their sequence homology, human GPCRs are

divided into five main families in the GRAFS classification system: glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin[3].

Their key roles in diverse cellular signalling pathways have made GPCRs major targets of many drugs. Identifying and elucidating the functions of GPCRs will provide opportunities for further drug discovery.

However, the GPCR signalling system in mammalians is elaborate, and using mammalian host cells to study GPCR signalling is relatively time-consuming. Functional characterisation of GPCRs and their mutants in mammalian expression systems is problematic because of the abundance of endogenous receptors for their ligands, and can be expensive. On the other hand, yeast cells provide a useful and relatively cost-effective model system in which to analyse GPCRs.

Description of the research domain

The budding yeast *S. cerevisiae* is an attractive host cell system for the study of GPCR signalling, for which only two pathways have been identified in this organism. One of the GPCR signalling pathways affects glucose sensing, whereas the other is involved in mating[8]. Glucose sensing requires the yeast G protein-coupled receptor-1 (Gpr1) and the G_{α} protein Gpa2, which is coupled to Gpr1. Activation of the receptor leads to production of cyclic adenosine monophosphate (cAMP), which – via protein kinase A (PKA) – stimulates diverse cell responses, such as growth, stress resistance, and storage of carbohydrates.

Mating is activated by α-factor pheromone and a-factor pheromone binding to the α-factor receptor (Ste2p) and a-factor receptor (Ste3p), respectively. Yeast of the a-cell type expresses a-factor pheromone and Ste2p, while the α-cell type expresses α-factor and Ste3p. During mating, pheromone binding to either receptor activates the MAPK pathway through the G_{α} protein Gpa1, which results in cell cycle arrest. Thus, haploid yeast cells encode a single endogenous GPCR (Ste2p or Ste3p) capable of activating the transduction pathway. The use of yeast as a host system prevents signalling cross-talk and activation of intrinsic GPCRs, as this endogenous GPCR in yeast can be deleted and replaced with

a mammalian GPCR. Additional advantages of using yeast systems to analyse GPCRs include their rapid growth, simplicity, low cost of culturing, and ability to be manipulated robotically.

For these reasons, yeast has proven a useful system for the study of human GPCRs. The first heterologous GPCR to be coupled to this pheromone pathway in yeast was the human β_2-adrenergic receptor (hβ-AR)[9]. In 1990, King and colleagues achieved this by replacing the yeast Ste2 gene with a modified hβ-AR gene, and placing it under control of the galactose-inducible GAL1 promoter. Induction with galactose yielded functional hβ-AR expression. However, interaction of Gpa1 in yeast appears to be efficient with only several human GPCRs. The subsequent creation of chimeric Gpa1 subunits, in which the C-terminal 5 amino acid residues of yeast Gpa1 are replaced by the equivalent residues of the mammalian G_α protein, advanced coupling to different receptors[10, 11]. The creation of this chimeric Gpa1/G_α transplant enabled cell-based functional yeast assays to be performed in a eukaryotic host free from endogenous responses.

Mutagenesis studies have been used to identify functionally important residues, motifs, and domains within the GPCR structure. Techniques to introduce mutations into GPCRs range from site-directed to random mutagenesis. In site-directed mutagenesis, selective point mutations are introduced to reveal the importance of the respective amino acid. Random mutagenesis produces large numbers of mutants, for which additional selection assays are required to identify the mutations of interest.

Incorporation of a reporter gene, such as green fluorescent protein (GFP) or lacZ, and growth selection marker HIS3, facilitates the detection of downstream G protein signalling. LacZ encodes β-galactosidase, which is responsible for cleavage of X-gal to yield a colored product. HIS3 encodes an enzyme that is involved in the production of histidine, an essential amino acid for yeast growth. In cell culturing, this allows the selection of cells which survive in histidine-deficient medium. When these reporter genes are put under the control of a pheromone-responsive promoter, such as FUS1 or FUS3, production of the transcripts of these genes could be coupled to the endogenous yeast GPCR

signalling pathway.

The combination of mutagenesis studies and human GPCR expression in yeast is applied in high-throughput approaches to identify agonists, antagonists, and allosteric modulators of both known GPCRs and orphan receptors. In this context, Beukers and IJzerman reviewed and highlighted the use of random mutagenesis combined with a functional screening assay in yeast, in 2005[12]. Considering their major contribution in signal transduction from ligand to effector via G proteins, GPCRs remain interesting targets for research in the present and future. Better understanding of the fundamental processes underlying ligand binding and receptor activation of GPCRs is of major importance for identifying potential targets for the development of new therapeutic agents.

Developments since 2005

In the past decade, yeast cells have served as a robust platform for structural and functional studies of human GPCRs. In this chapter, discoveries since 2005 concerning different family members of human GPCRs will be described.

Adenosine receptors

Adenosine, a purine nucleoside, is a key endogenous molecule in regulation of immune and inflammatory systems[13]. The purine nucleoside is found in low levels in unstressed, healthy tissues. Under conditions of metabolic stress and cell damage, adenosine is accumulated in the extracellular space. Adenosine is capable of activating four different adenosine receptors: A_1, A_{2A}, A_{2B}, and A_3. All the adenosine receptors are ubiquitously expressed throughout the human body[14].

Adenosine A_1 receptor

Stewart and colleagues used yeast as a system to determine the relative efficacies of agonists and affinity values for both agonists and antagonists of the adenosine A_1 receptor[15]. For this purpose, *S. cerevisiae* strains expressing chimeric Gpa1/

G_{α} were transformed with a human adenosine A_1 receptor construct. Chimeras of Gpa1/$G_{\alpha q}$, Gpa/$G_{\alpha 12}$, Gpa1/$G_{\alpha o}$, Gpa1/$G_{\alpha i1/2}$, Gpa1/$G_{\alpha 3}$, and Gpa1/$G_{\alpha 5}$ were used in combination with the lacZ reporter gene, under the control of the FUS1 promoter. β-Galactosidase activity was measured using a fluorescent substrate to determine the ability of the agonists R-PIA and VCP-189 to elicit a response when coupled to an individual subtype of G protein. Results of these yeast signalling assays revealed that R-PIA was a high-efficacy agonist when coupled to $G_{\alpha o}$, $G_{\alpha i1/2}$, or $G_{\alpha i3}$ (Fig. 1, ●), whereas VCP-189 was a lower-efficacy partial agonist when coupled to $G_{\alpha i}$ proteins (Fig. 1, ▲). Validation of $G_{\alpha i/o}$ coupling preferences was further performed in Chinese hamster ovary cells expressing the human A_1 receptor.

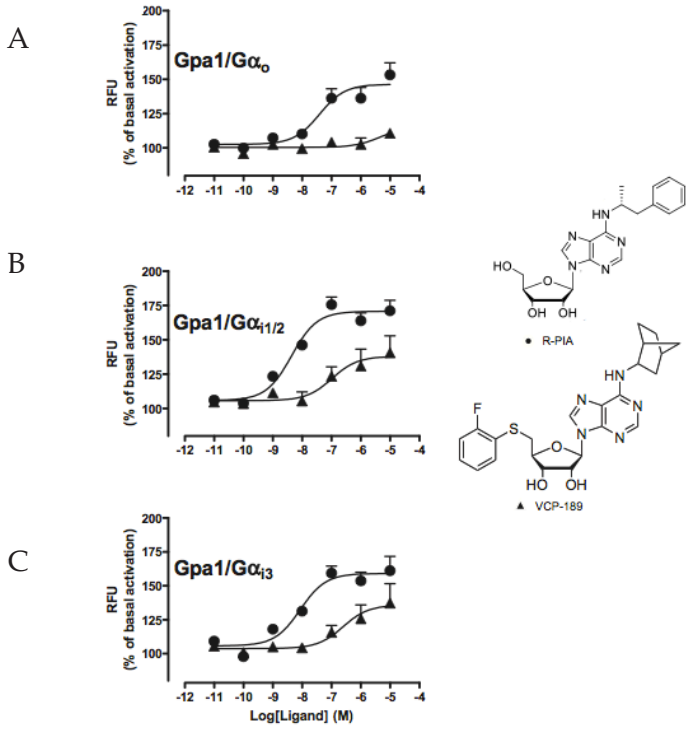

Fig. 1. Influence of G protein subtype on R-PIA and VCP-189 response. Yeast strains expressing the adenosine A_1 receptor with Gpa1/$G_{\alpha o}$ (A), Gpa1/$G_{\alpha i1/2}$ (B), or Gpa1/$G_{\alpha i3}$ (C) were incubated with a range of ligand concentrations for 18 to 24h. Ligand response is expressed in relative fluorescence unit (RFU) corrected for the fluorescence in the absence of ligand. Data points represent mean ± SEM obtained from three to five experiments performed in duplicate. *Reproducted with permission from* [15].

In addition to the G_α subunit, domains in the adenosine A_1 receptor itself also influence signalling activity. Within the GPCR structure, the intracellular and extracellular loop regions that connect the seven transmembrane domains are highly variable in length and sequence. In this context, the role of the second and third extracellular loops of the human adenosine A_1 receptor in activation and allosteric modulation was examined[16]. The *S. cerevisiae* expression vector containing the human adenosine A_1 receptor gene was used for mutagenesis. The second extracellular loop (EL2) was subjected to an $(Ala)_3$-scan, in which consecutive sets of three amino acids were replaced by alanine residues. On EL2 and the third extracellular loop (EL3), site-directed mutagenesis was performed too by introducing single alanine mutations to the loops. Plasmids containing the human adenosine A_1 receptor gene were transformed into a *S. cerevisiae strain*, which harbors chimeric Gpa1/$G_{\alpha i3}$ and is coupled to the FUS1-HIS3 reporter gene system. As an indicator of receptor activity, yeast growth in histidine-deficient medium was observed for the mutant receptors after agonist stimulation. In radioligand binding experiments, affinity of the mutant receptors for agonist CPA in the presence of radiolabeled antagonist DPCPX was explored. To investigate the sensitivity of the mutants to allosteric modulation, radioligand binding experiments were performed with agonist CPA in the presence of allosteric modulator PD81,723. Results of this study revealed the importance of many residues in the second and third extracellular loops in human adenosine A_1 receptor activation, since single point mutations affected the potency of CPA for the adenosine A_1 receptor. $W156^{EL2}$ and $E164^{EL2}$ were identified to be involved in allosteric modulation. After mutation to alanine in $W156^{EL2}$, PD81,723 lost its ability to enhance CPA potency, whereas mutation of $E164^{EL2}$ increased the effect of PD81,723 on CPA affinity. These data suggest that the binding site for PD81,723 is located in or close to the extracellular domains of the A_1 receptor.

Adenosine A_{2B} receptor

In chapter 3 of this thesis, we analysed the interaction between the low affinity adenosine A_{2B} receptor and the C-terminus of G_α subunits[17]. Plasmids containing the wild-type and mutant human adenosine A_{2B} receptor were transformed into

engineered *S. cerevisiae* yeast strains expressing different Gpa1/G_{α} chimeras. The FUS1-HIS3 reporter gene system enabled determination of G protein coupling and activation via observation of yeast growth after exposure to agonist 5'-*N*-ethylcarboxamido-adenosine (NECA). A tyrosine at the C-terminus of the G_{α} subunit was revealed to be essential in controlling A_{2B} receptor activation. In the receptor, R103$^{3.50}$ and I107$^{3.54}$ mutation to alanine eliminated receptor activation, whereas L213^{IL3} mutation to alanine improved coupling efficiency in all yeast strains. The S235$^{6.36}$A mutant receptor showed the most divergent humanized G protein coupling profile and substitution of L236$^{6.37}$ decreased receptor activation in all yeast strains representing the different G protein pathways. In chapter 4 of this thesis, we used the same 'a single-GPCR-one-G protein' yeast system to screen for key residues within the NPxxY(x)$_6$F motif and in helix 8 of the adenosine A_{2B} receptor[18]. Each residue was mutated to alanine through site-directed mutagenesis. Four mutants P287$^{7.50}$A, Y290$^{7.53}$A, R293$^{7.56}$A and I304$^{8.57}$A showed a complete loss of function.

Li et al. were the first to describe inverse agonists for the human adenosine A_{2B} receptor. Constitutively active mutants (CAMs) are mutant receptors that show basal activity independent of an agonist, and provide a useful tool to discriminate inverse agonists from neutral antagonists. The use of CAMs with varying levels of constitutive activity enabled determination of differences in relative intrinsic efficacy values of the three inverse agonists ZM241385, DPCPX, and MRS1706[19]. For this purpose, an engineered *S. cerevisiae* strain expressing chimeric Gpa1/$G_{\alpha i3}$ in combination with the FUS1-HIS3 reporter gene system was used. Nine CAMs were identified from a random mutation bank using yeast growth assays, which were used as screening tools to discover inverse agonists. The three compounds ZM241385, DPCPX, and MRS1706 were able to inactivate the constitutively active human adenosine A_{2B} receptor mutants, thus acting as inverse agonists on these CAMs. Consequently, these three compounds were defined as inverse agonists on the human adenosine A_{2B} receptor. The effects of the three inverse agonists on the A_{2B} receptor were dependent on the level of constitutive activity of the mutants. Two high-level CAMs appeared locked in an active state and were insensitive to the three inverse agonists. Three intermediate-

level CAMs were partially inhibited, whereas four low-level CAMs were almost completely inhibited.

The role of distinct domains within the adenosine A_{2B} receptor was examined in several studies. The first extracellular loop was selected to perform successive mutagenesis experiments to analyze changes in agonist potency, constitutive activity, and intrinsic activity[20]. Plasmids containing the human adenosine A_{2B} receptor gene were transformed into an *S. cerevisiae* strain, which was engineered to express chimeric Gpa1/$G_{\alpha i3}$. As mentioned before, the use of the FUS1-HIS3 reporter gene system enabled growth as a readout for receptor activity. First, random mutations were introduced in the fragment encoding for the first three transmembrane domains, the first extracellular loop, the first intracellular loop, and part of the second intracellular loop of the human A_{2B} receptor. The random mutagenesis screen revealed the involvement of $F71^{EL1}$ and $D74^{EL1}$ in receptor activation. Site-directed mutagenesis on these residues, and a subsequent yeast growth assay showed altered potency of agonist NECA. Additionally, the mutant receptors responded to the inverse agonist ZM241385 which suppressed constitutive activity. Then, site-saturation mutagenesis was performed on the respective positions in the first extracellular loop of the GPCR. Phenylalanine substitution at position 71 by serine, arginine, alanine, or glycine resulted in the largest decrease in potency and maximum intrinsic activity. Activation profiles of aspartic acid substitution at position 74 showed large variations. Remarkably, substitution at this position by glutamine, glutamic acid, asparagine or serine caused an increase in potency and constitutive activity. Radioligand binding assays revealed that almost all mutated receptors showed similar binding of the antagonist PSB603, indicating a similar expression level.

Using the same yeast strain, MMY24, random mutagenesis was performed on the fragment encoding the extracellular loop 2 (EL2) and its two adjacent transmembrane domains (TM4 and TM5) of the human adenosine A_{2B} receptor. By simply changing the concentration of the histidine synthesis inhibitor 3-AT and the introduction of a specific time frame, Peeters et al. mapped the residue locations of constitutively active mutants (CAMs)[20] and constitutively inactive mutants (CIMs) of the TM4-EL2-TM5 area[21]. For CAMs screening, 1 nM NECA

was used with 7 mM 3-AT and clones were picked up after three days. This yielded 12 different CAM mutant receptors with increased agonist potency containing mutations located at the top of TM4, in a cysteine-rich region of EL2, and at the bottom half of TM5. Among the mutant receptors, residues F141$^{4.61}$, C167^{EL2}, and Y202$^{5.58}$ were found to be mutated multiple times, indicating an essential role of these residues in A_{2B} receptor activation mechanisms. Substitution of Y202$^{5.58}$ by an asparagine residue resulted in a 38-fold increase as compared to the wild-type receptor. Substitution of F141$^{4.61}$ with leucine resulted in a 25-fold increase in potency for NECA compared with the wild-type receptor, as assessed by yeast growth assays. Moreover, radioligand binding assays revealed a 13-fold increase in NECA affinity compared with the wild-type receptor, suggesting an essential role of F141$^{4.61}$ in agonist binding. The CIM screening does not require the presence of an agonist or inverse agonist. At 1 mM 3-AT clones were picked up after 4 days and 6 days, identifying 22 CIMs containing a total of 25 different mutated amino acid positions that showed a decrease in constitutive activity as well as in agonist potency. Most CIMs were found in the top half of TM5 without a single CAM. However, a cluster of CAMs is found in EL2 without containing any CIMs. Interestingly, six residues, I136$^{4.56}$, F141$^{4.61}$, S146^{EL2}, T155^{EL2}, F173^{EL2} and Y202$^{5.58}$ were found in both CAM and CIM screening. These residues are most likely crucial players in activation or inactivation of the adenosine receptor. In this perspective CIMs and CAMs could correspond to the receptor in different conformational states.

Adenosine A_{2A} receptor

O'Malley et al. demonstrated a method for the production, purification, and characterisation of the human adenosine A_{2A} receptor[22]. For this purpose, human A_{2A} receptor, whether or not fused to GFP, was re-engineered with a C-terminal deca-histidine tag, and plasmids containing this vector were transformed into a *S. cerevisiae* strain. Facilitated by the presence of the attached GFP, yeast cells were screened for their ability to express the tagged A_{2A} receptor, and subsequently lysed to solubilise the A_{2A} receptor. Several purification steps, which included immobilized metal affinity chromatography, yielded purified and stabilised A_{2A}

receptor. Sufficient quantities of the receptor were obtained for spectroscopic studies, which confirmed that the A_{2A} receptor structure is largely α-helical.

For a better understanding of why many receptors improperly traffick or are inactive in heterologous expression systems, expression of 12 human GPCRs in *S. cerevisiae* was investigated[23]. Plasmids containing one of these 12 human GPCRs from the rhodopsin family, and an additional GFP-tag, were transformed into a yeast strain. Of these GPCRs, only the adenosine A_{2A} receptor was active in terms of ligand binding and was located primarily at the plasma membrane, as observed by radioligand binding assays and confocal imaging, respectively. The extent of leader sequence cleavage, which reflects processing and trafficking of the expressed GPCRs, was determined through a mobility shift experiment of GFP-tagged receptors. The A_{2A} and A_{2B} receptor were the only receptors to demonstrate both leader sequence processing and activity, indicating that translocation is a critical limiting step in the production of active human GPCRs in *S. cerevisiae*.

The yeast system is also used for screening thermostabilising mutations, which can improve the overall stability of receptor proteins to eventually obtain high-resolution crystal structures of GPCRs. Analysis of the mutations alone and in combination using the yeast assay, together with the known $A_{2A}R$ crystal structures provides information on the role of individual amino acids in receptor function. Bertheleme et al. found $R199^{5.60}$ and $L208^{IL3}$ play key roles in A_{2A} receptor function using the yeast cell growth assay in the MMY24 strain[24], as these mutations abolished constitutive activity of the A_{2A} receptor; In contrast, the $F79^{3.31}A$ mutation increased constitutive activity, potency and efficacy of the receptor, most likely at the expense of receptor stability.

Somatostatin receptor 5

The somatostatin receptor 5 (SSTR5) is a member of the γ-group of rhodopsin receptors in the GRAFS classification system of GPCRs[3]. Somatostatin is a cyclic neuropeptide known as a growth hormone release-inhibiting factor. Somatostatin regulates the human endocrine system by binding to somatostatin receptors (SSTRs), of which five subtypes have been identified: SSTR1-SSTR5[25,26]. Among

these subtypes, SSTR2 and SSTR5 predominantly regulate growth hormone secretion in acromegaly patients, and are expressed in the majority of growth hormone secreting tumors[27].

Several research groups used the human SSTR5 receptor as a model for a human GPCR. Iguchi et al. performed site-directed mutagenesis of asparagine residues on the two N-linked glycosylation motifs in the extracellular N-terminal domain of human SSTR5[28]. These Asn residues had been shown to be important for ligand affinity, and for plasma membrane localisation and signalling, as mutation of the Asn residues affected somatostatin ligand binding and adenylate cyclase activity in rat[29]. In the mutagenesis study, *S. cerevisiae* was used as a host to examine the effects of substitution of the $N13^{N\text{-term}}$ and $N26^{N\text{-term}}$ residues by alanine residues[28]. Enhanced green fluorescent protein (EGFP) was fused to the human SSTR5. Results showed the importance of these asparagine residues of the human SSTR5 receptor in signalling, since substitution significantly decreased signalling activity.

For agonist detection of human GPCRs, researchers continued to use SSTR5 as a model receptor[30]. The strategy of a feedback signal activation system was used to analyse agonist stimulation of GPCRs. It was aimed to express both the GFP reporter gene and G_β after G protein activation by agonist binding to a given GPCR. A *S. cerevisiae* strain with overexpression of the STE4 gene, which encodes for the yeast G_β, under the control of FIG1 promoter, was created. Overexpression of G_β was expected to result in the generation of free $G_{\beta\gamma}$ dimers, as a consequence of competing with endogenous γ-associated β subunits for binding to G_α. The free $G_{\beta\gamma}$ dimers were able to amplify the human yeast-coupled G protein signal. This feedback signal activation system resulted in MAPK cascade activation by $G_{\beta\gamma}$, and subsequent amplification of GFP expression. This high-sensitivity method for the detection of agonist activity of human GPCRs in yeast was applied to human SSTR5. Therefore, IMFD-70 and IMFD-70B yeast strains were transformed with plasmids expressing human SSTR5, exposed to different concentrations of somatostatin, and subjected to a transcription assay using the GFP fluorescent reporter gene. GFP fluorescence intensity induced by human SSTR5 in the feedback activation strain (IMFD-70B; Fig. 2, ●) was higher

than in the common activation strain (IMFD-70; Fig. 2, ▲) at all somatostatin concentrations tested. Interestingly, fluorescence intensity induced by human SSTR5 in the common activation strain (Fig. 2, ▲) was found to be lower compared with the fluorescence intensity induced by Ste2p (Fig. 2, Δ), whereas the intensities of the Ste2p (Fig. 2, ○) and SSTR5 (Fig. 2, ●) feedback activation strain were equivalent. These results suggest an improvement of agonist signal detection and SSTR5 response sensitivity by feedback signal activation.

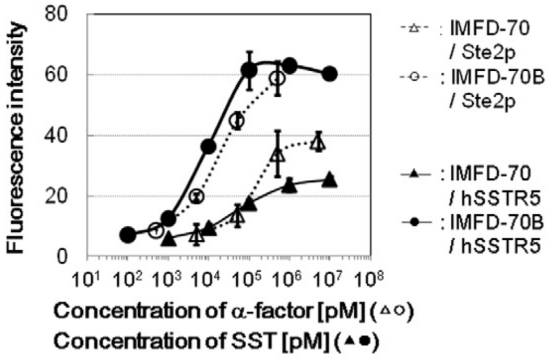

Fig. 2. Concentration-response curves of common and feedback activation strains of yeast expressing human SSTR5 in response to somatostatin. Yeast strains were transformed with plasmids containing human SSTR5, grown in SDM71 medium, and incubated with different concentrations of somatostatin for 18h. Fluorescence intensity of common activation strain (IMFD-70/hSSTR5) and the feedback signal activation strain (IMFD-70B/hSSTR5) in response to increasing somatostatin concentrations are displayed. The concentration-response curves of the corresponding yeast strains harboring Ste2p are presented by dashed lines. Data points represent fluorescence intensities of 10,000 cells defined as the geometric mean ± standard deviation (*n = 3*). *Reproduced with permission from* [30].

Researchers from the same department demonstrated somatostatin-specific signalling functions of human SSTR5 by addition of prepro- and pre-regions of α-factor, and a 20 amino acid N-terminal signalling sequence of yeast Ste2 (Ste2N) to the N-terminus of human SSTR5[31]. Additionally, the C-terminal 5 amino acid residues of Gpa1 were replaced by the equivalent residues of the human $G_{\alpha i3}$ to create a chimeric Gpa1/$G_{\alpha i3}$ transplant. Then, a yeast-based fluorescent signalling assay system that expresses the GFP reporter gene was used for evaluation of signalling activity of SSTR5 and response to somatostatin binding. Since the potency of somatostatin for native SSTR5 was lower than for

Ste2N-SSTR5, introduction of this yeast signal sequence apparently increased ligand sensitivity. Lastly, SSTR5 expression levels and localisation were examined using a cyan fluorescent protein tag, fused to the SSTR5 C-terminus. Ste2N-SSTR5 seemed to improve localisation to the plasma membrane as well, suggesting that SSTR5 was transported by different protein sorting pathways in the presence of the signalling sequence.

To improve identification of signal promotion by $G_{\alpha i}$-specific human GPCRs using flow cytometry, SSTR5 served as a model for heterologous GPCRs expressed in engineered *S. cerevisiae* yeast cells[32]. For this purpose, human SSTR5 was expressed from a multicopy episomal plasmid under the control of the PGK1 promoter, for substitution of yeast Ste2p. Chimeric Gpa1/$G_{\alpha i3}$ was used as replacement of yeast Gpa1. The GFP gene was again used as a reporter under the control of the FIG1 promoter, and somatostatin was added to the engineered yeast cells. To determine SSTR5 signalling via $G_{\alpha i}$, GFP fluorescence was measured using flow cytometry. In addition, several genes in yeast were deleted to improve flow cytometric separation. Sst2p, a Gpa1-specific GTPase-activating protein, was deleted to confer hypersensitivity to agonists. The FAR1 gene, which encodes a cyclin-dependent kinase inhibitor and mediates cell cycle arrest, was deleted to allow the recovery of episomal plasmids from signal-activated yeast cells, which is important for extensive screening. The constructed IMFD-72 strain, which contains the mentioned alterations, enabled improved flow cytometric separation to identify signal transduction mediated by the human SSTR5.

Recently, Nakamura and colleagues developed a method to simultaneously detect dimerisation and signalling of GPCRs in *S. cerevisiae* by a dual-color reporter system[33]. Two fluorescent proteins with distinct emission peaks, EGFP and DsRed-Express2 (E2-Crimson), were expressed under the control of the PGK1 promoter. Fluorescence signals were measured in yeast cells harboring these plasmids, and could be completely distinguished by flow cytometry. For the split-ubiquitin two-hybrid system, yeast strains were constructed to express E2-Crimson in response to the release of artificial transcription factor LexA-VP16 by ubiquitin-specific proteases. These proteases

recognize the NubG/Cub complex, which is formed by fusion of the proteins to which these two split tags (NubG and Cub) are attached. Based on this principle, this method permits detection of GPCR dimerisation. The yeast-based signalling assay, using EGFP, to study GPCR signalling was combined with the split-ubiquitin two-hybrid system to detect dimerisation and signalling simultaneously. The technique was validated by monitoring simultaneous homo- and hetero-dimerisation and somatostatin-induced signalling in human SSTR5. For this purpose, yeast strains co-expressing SSTR5-Cub-LexA-VP16 and various GPCR-NubG were exposed to somatostatin, and fluorescence was measured by flow cytometry (Fig. 3). Regarding GPCR dimerisation, cells co-expressing SSTR5-Cub-LexA-VP16 and SSTR2-NubG or ADRB2-NubG showed E2-Crimson fluorescence, while cells co-expressing SSTR5-Cub-LexA-VP16 and Ste2p-NubG and the mock control cells did not show E2-Crimson fluorescence (Fig. 3A). Regarding GPCR signalling, all cells displayed somatostatin-induced EGFP fluorescence, without significant difference between the groups (Fig. 3B). According to these results, SSTR5 could heterodimerise with SSTR2 and the β_2-adrenergic (ADRB2) receptor, which has no effect on signalling activity after somatostatin exposure.

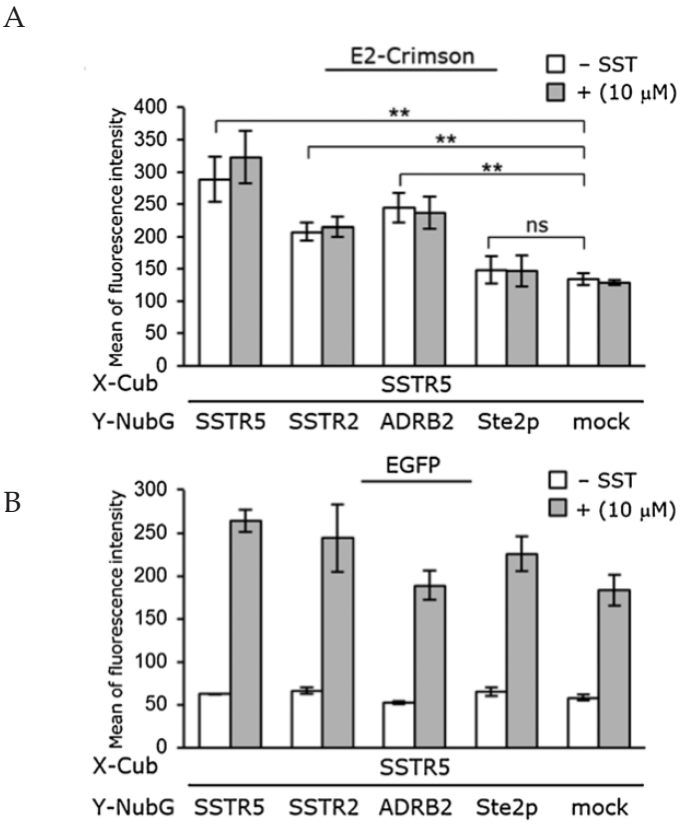

Fig. 3. Simultaneous detection of dimerisation and signalling by human SSTR5. Yeast strains were transformed with plasmids encoding various GPCR-NubG constructs ("Y-NubG") and SSTR5-Cub-LexA-VP16 ("X-Cub"), grown in SD selective medium for 18h, and incubated with or without 10μM somatostatin for 4h. Mock cells co-expressing SSTR5-Cub-LexA-VP16 and NubG served as negative control. E2-Crimson (A) and EGFP (B) fluorescence intensities of 10,000 cells were measured by flow cytometry. Values represent mean ± standard deviation. Statistical significance was assessed by the t-test (** $p < 0.01$; ns, not significant). SSTR5, somatostatin receptor 5; SSTR2, somatostatin receptor 2; ADRB2, β2-adrenergic receptor; Ste2p, yeast α-factor receptor. *Reproduced with permission from* [33].

Muscarinic acetylcholine receptor

The muscarinic acetylcholine receptors (mAChRs) are members of the biogenic amine receptor cluster of the α-group of rhodopsin receptors, according to the GRAFS classification system of GPCRs[3]. Acetylcholine, the first neurotransmitter identified, is released from vesicles in presynaptic nerve terminals and binds to either nicotinic (ion channels) or muscarinic receptors (GPCRs). Five subtypes of muscarinic acetylcholine receptors, M_1-M_5, have been

identified in human[34]. M_1, M_4, and M_5 mAChRs are predominantly expressed in the central nervous system, whereas M_2 and M_3 subtypes are expressed widely in the central nervous system and periphery[35, 36]. In the central nervous system, mAChRs regulate important central functions, including cognitive, behavioural, sensory, motor and autonomic processes. Peripheral mAChRs mediate acetylcholine-mediated decreases in heart rate, and increases in smooth-muscle contractility and glandular secretion. The muscarinic acetylcholine receptors are implicated in a variety of human diseases, including Alzheimer's disease, overactive bladder disorder, and chronic obstructive pulmonary disease[37].

Different *S. cerevisiae* strains expressing different Gpa1/G_{α} chimeras were coupled to the lacZ reporter gene for functional readout of M_3 mAChR ligands, previously established as orthosteric antagonists in mammalian cells[38]. The yeast strains were transformed with a vector containing the gene encoding a modified rat M_3 mAChR. Using this yeast platform, "antagonists" atropine and pirenzepine were found to be inverse agonists when the receptor was coupled to Gpa1/$G_{\alpha q}$, and low efficacy agonists when coupled to Gpa1/$G_{\alpha 12}$. This indicates functional selectivity at the M_3 mAChR. To validate these findings, studies with atropine were continued in mammalian 3T3 mouse embryonic fibroblasts expressing full-length human M_3 mAChR.

In an extended study, the applicability of this yeast platform to identify allosteric ligand-mediated functional G protein selectivity was tested[39]. For this purpose, the effect of brucine on carbachol affinity at the M_3 receptor was investigated. Results indicate that brucine is a partial allosteric agonist and positive modulator of carbachol when coupled to Gpa1/G_q, a positive modulator when coupled to Gpa1/G_{12}, and a neutral modulator when coupled to Gpa1/$G_{\alpha i1/2}$. For validation, human M_3 mAChR was further expressed in Chinese hamster ovary cells.

More recently, the X-ray crystal structure of active agonist-bound human M_2 mAChR was solved, and stabilised by a G protein mimetic nanobody for the M_2 receptors[40]. In this study, a *S. cerevisiae* strain was used for the conformational selection of these nanobodies for enhancement of the affinity of M_2 for its agonist iperoxo. Several selection rounds were performed with a post-

immune single variable domain nanobody complementary DNA library, which was constructed by PCR amplification and transformation into the yeast strain. Additionally, the X-ray crystal structure was solved for the M_2 receptor bound to both iperoxo and positive allosteric modulator LY2119620 in this research[40].

In another research group, Suharni et al. used *S. cerevisiae* as expression system to identify the epitope scFV 18A107 located in IL3 of the M_2 receptor[41]. A selected recombinant antibody fragment turned out to be highly specific for recognizing the M_2 receptor, because the amino acid sequence of scFV 18A107 does not exist in other mAChRs.

Neurotensin receptor type 1

The neurotensin receptor type 1 is a high-affinity neurotensin receptor, and belongs to the α-group of rhodopsin receptors in the GRAFS classification system for GPCRs[3]. Neurotensin is a tridecapeptide that acts as a neuromodulator of dopamine transmission in the nigrostriatal and mesocorticolimbic system of the central nervous system[42]. Peripherally, neurotensin plays a role in hormonal and neurocrine regulation in the gastrointestinal tract, including inhibition of small bowel motility and gastric acid secretions, and facilitation of fatty acid absorption[43]. Neurotensin exerts its effects by binding to neurotensin receptor type 1 (NTSR1), NTSR2, and NTSR3. Abnormal expression of NTSR1 during the early stages of cell transformation in relation with Wnt/β-catenin pathway deregulation is presumed to induce carcinogenesis[44, 45]. NTSR1 expression could serve as a prognosis marker in various types of cancer.

To monitor the activation of human NTSR1 signalling, a fluorescence-based microbial yeast biosensor was constructed[46]. Microbial *S. cerevisiae* strains IMFD-70, IMFD-72, and IMFD-74 were transformed with plasmids expressing human NTSR1. The constructed IMFD-74 strain expresses Gpa1/$G_{\alpha q}$ and harbors the same alterations as the IMFD-72 strain, in which the Gpa1/$G_{\alpha i3}$ transplant is expressed[32], whereas the IMFD-70 strain expresses intact Gpa1. GFP reporter gene under the control of FIG1 promoter was used to sense NTSR1 signalling in response to neurotensin peptide stimulation. The IMFD-74 (Fig. 4, □) strain induced higher fluorescence above 1 μM neurotensin peptide and relatively

lower fluorescence below 10 nM neurotensin peptide when compared to the IMFD-72 strain (Fig. 4, ∆). Moreover, the fluorescence levels induced by these two yeast strains, were higher than the fluorescence levels of IMFD-70 (Fig. 4, ○) at all concentrations of neurotensin peptide. According to these results, the use of the IMFD-74 yeast strain permitted convenient flow cytometric sensing of human NTSR1 signalling, induced by neurotensin, in yeast.

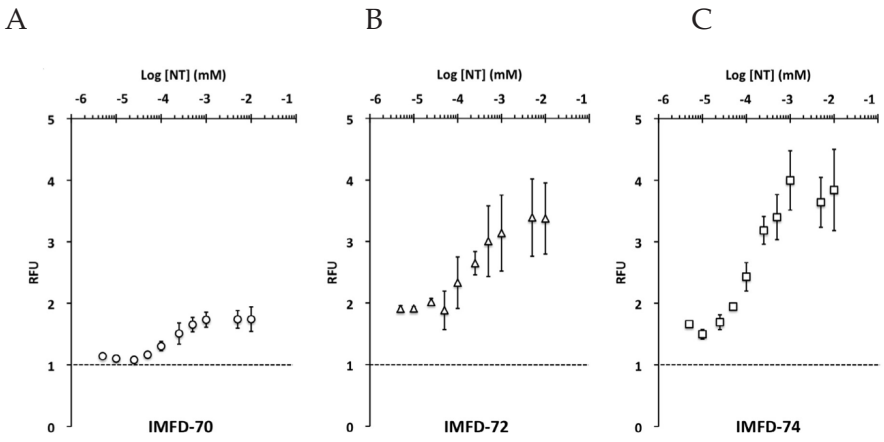

Fig. 4. Concentration-response curves for NTSR1 signalling after exposure to neurotensin peptide. Yeast strains IMFD-70 (A), IMFD-72 (B), and IMFD-74 (C) were incubated in SD selective medium containing various concentrations of neurotensin peptide for 10h. Data points represent relative fluorescence unit (RFU) ± standard deviation (*n = 3*). *Reproduced with permission from* [46].

Angiotensin II type 1 receptor

Angiotensin II (Ang II) type 1 receptor is a member of the rhodopsin receptors in the GRAFS classification system of GPCRs[3]. Ang II represents the primary effector molecule of the renin-angiotensin system, thus contributing to cardiovascular homeostasis[47, 48]. Ang II binds to two major subtypes of Ang II receptors, Ang II type 1 receptor (AGTR1) and Ang II type 2 receptor (AGTR2). However, most of the known physiological effects of Ang II are mediated by AGTR1, which is expressed in a variety of organ systems, including the heart, kidney, and brain[48].

Recently, Nakamura and colleagues investigated the functional activation of AGTR1-mediated signalling in yeast cells[49]. Interaction of $N295^{7.46}$ with $N111^{3.35}$

was suggested to play a role in determining the peptide binding selectivity of AGTR1 receptors[50, 51]. Therefore, a single alanine or serine mutation was introduced at $N295^{7.46}$ of human AGTR1, and the chimeric Gpa1/$G_{\alpha i3}$ transplant was used to enable functional activation of AGTR1 in *S. cerevisiae*. In this IMFD-72 strain, *Zoanthus* sp. green fluorescent protein (ZsGreen) reporter gene, under the control of the FIG1 promoter, was incorporated. The resulting IMFD-72ZsD yeast strain was exposed to Ang II and Ang II peptidic analogs which differ in affinity towards AGTR1. This resulted in successful signal transmission inside the yeast cell, as monitored using a fluorescence reporter assay. Subsequently, the IMFD-72ZsD yeast strains were transformed with the human $N295^{7.46}$-mutated ($N295^{7.46}$A and $N295^{7.46}$S) AGTR1 expression plasmids and peptide (Ang II and Ang III) expression plasmids. The yeast strain expressing AGTR1-$N295^{7.46}$A (Fig. 5, □) or AGTR1-$N295^{7.46}$S (Fig. 5, ■) and Ang II showed a 94-fold and 91-fold increase in ZsGreen fluorescence intensity, respectively, compared with the mock strain. Ang III induced a 36-fold and 45-fold increase in ZsGreen fluorescence intensity compared with the mock strain, for $N295^{7.46}$A (Fig. 5, □) or $N295^{7.46}$S (Fig. 5, ■), respectively. These results suggest that autocrine Ang II peptide and its analog, Ang III, produced and secreted by engineered yeast cells, can themselves promote AGTR1-mediated signalling.

C-X-C chemokine receptor type 4

CXCR4 is a member of the chemokine cluster of the γ-group of rhodopsin receptors, as classified by GRAFS[52]. Receptors for different types of chemokines (CC, CXC, C, and CX3C) are G protein-coupled receptors[53]. Whereas redundancy exists in the repertoire of chemokine- and receptor-binding activities, stromal cell-derived factor 1 (SDF-1/CXCL12) is the sole ligand for C-X-C chemokine receptor type 4 (CXCR4)[54]. CXCR4 is expressed on human hematopoietic stem cells, and plays a critical role in the development of B lymphocytes, the chemotaxis of lymphocytes, granulocytes and other inflammatory cells[52]. CXCR4 was the first coreceptor identified that was permissive for fusion and entry of human immunodeficiency virus type 1 (HIV-1) into $CD4^+$ human target

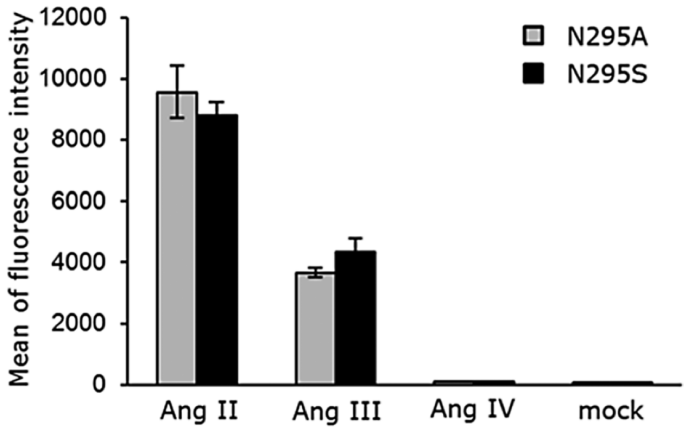

Fig. 5. Activation of human AGTR1 by agonistic peptides produced and secreted by the engineered IMFD-72ZsD yeast strain. Yeast strain IMFD-72ZsD was transformed with the human N295$^{7.46}$-mutated (N295$^{7.46}$A and N295$^{7.46}$S) AGTR1 expression plasmids and peptides (Ang II, Ang III and Ang IV) expression plasmids, incubated in SD selective medium for 9 h, and ZsGreen fluorescence was measured by flow cytometry. The mock strain was transformed with a plasmid lacking peptide expression, and served as negative control. Values represent fluorescence intensities of 10,000 cells defined as mean ± standard deviation ($n = 3$). *Reproduced with permission from* [49].

cells[55]. In addition, CXCR4 is expressed by tumor cells of many types of malignancies, and is implicated in cancer metastasis[56].

Yeast was used as host system in a series of experiments with the human CXCR4[57]. Wild-type and constitutively active mutant human CXCR4s were expressed in an *S. cerevisiae* strain coupled to reporter gene HIS3 or lacZ under the control of the FUS1 promoter. Using the FUS1-HIS3 reporter gene system, constitutively active CXCR4 mutants were selected after exposure to CXCL12 and subsequent evaluation of growth survival. Then, the FUS1-lacZ reporter gene system was introduced into the engineered yeast cells expressing human wild-type and mutant CXCR4 to perform screening assays. β-Galactosidase activity after exposure to compounds derived from T140, an inverse agonist for CXCR4, was measured using a fluorescent substrate. Alternatively, the FUS1-FUI permease reporter gene system was applied in a growth assay for characterization of inverse agonist FC131. Since FUI is a permease for 5-fluorouridine (5-FU), CXCR4-mediated activation of the pheromone response upregulates permease expression and sensitivity to 5-FU toxicity.

Complement factor 5a receptor

Complement factor 5a receptor (C5aR) is a rhodopsin-like GPCR, expressed on the surface of neutrophils and other myeloid cells. Complement factor 5a is a 74-amino acid peptide released during complement activation[58]. Binding to C5aR initiates neutrophil chemotaxis and release of proteolytic enzymes and superoxide.

The contribution of the first and third extracellular loops of C5aR in C5aR activation was investigated by subjecting these loops to random saturation mutagenesis[59]. Random amino acid substitutions were introduced into the first (EL1) and third (EL3) extracellular loops of C5aR. The resulting mutant receptors were subcloned into an ADE2 yeast expression vector. In the *S. cerevisiae* strain, yeast Gpa1 was replaced by chimeric Gpa1/$G_{\alpha i3}$, and coupled to reporter gene HIS3 under the control of the FUS1 promoter. The cells of this yeast strain were transformed with the EL1 and EL3 ADE2 libraries. Growth of yeast cells in histidine-deficient medium while coexpressing C5a ligand allowed the identification of functional receptors. To determine receptor signalling through activation of the MAPK pathway, yeast cells were incorporated with the lacZ reporter gene and transformed with the EL1 and EL3 libraries. After stimulation with agonist W5Cha, β-galactosidase activity was measured to determine the effect of a certain mutation in the EL1 or EL3 of C5aR on receptor function. Results provided proof for an essential WXFG motif in the first extracellular loop, as mutations in this motif disrupted receptor activation. However, C5a binding remained unaffected by mutations in the first extracellular loop of C5aR, indicating a role for the WXFG motif downstream of ligand binding.

Rana and Baranski performed a study to explore the role of EL3-N-terminus conserved cysteine pairs on receptor conformation and G protein activation[60]. The *S. cerevisiae* strain, harboring a chimeric Gpa1/$G_{\alpha(1/2)}$ as replacement of yeast Gpa1, was coupled to the FUS1-lacZ reporter gene. They first investigated wild-type and mutant CXCR4 plasmids, containing point mutations at cysteine residues, which were transformed to yeast and exposed to CXCL12. Mutations of $C28^{N\text{-}term}$ and $C274^{EL3}$ with a serine pair ($C28^{N\text{-}term}S$/ $C274^{EL3}S$) abrogated CXCR4 receptor activation. Introduction of either a salt

bridge pair ($C28^{N\text{-}term}R/C274^{EL3}E$) or an aromatic pair ($C28^{N\text{-}term}F/C274^{EL3}F$) restored CXCL12-mediated signalling.

For further investigation of the role of cysteine pairs, an artificial EL3-N-terminus cysteine pair ($C30^{N\text{-}term}$ and $C272^{EL3}$) was engineered into a novel constitutively active mutant ($N119^{3.35}S$ mutation) of C5aR, which originally had serine residues at these positions. This resulted in restrained constitutive signalling without affecting C5a-induced activation, as assessed by monitoring β-galactosidase activity. Single $S272^{EL3}C$ mutation resulted in impaired C5a-induced signalling, which could be rescued by introduction of another cysteine in the N-terminus ($S30^{N\text{-}term}C$ mutation). Additional mutational studies demonstrated that substitution of $S272^{EL3}$ with alanine or threonine impaired C5a-induced signalling too. The additional $S30^{N\text{-}term}C$ mutation did not affect constitutive signalling or C5a-induced signalling. However, the $S30^{N\text{-}term}C$ mutation rescued C5a-mediated signalling in wild-type C5aR, indicating that a covalent disulfide bond is not required for the rescue effect. These results indicate a possible noncovalent interaction between EL3 and the N-terminus in C5aR.

Hedgehog signalling receptor, Smoothened

The hedgehog signalling receptor, smoothened (SMOH), belongs to the frizzled/taste2 receptor family of the GPCRs[3]. The Hedgehog (Hh) signalling pathway regulates organ development during embryogenesis[61]. In adults, the pathway is reactivated by tissue damage and stimulates tissue repair, including peripheral nerve regeneration[62, 63]. Hh protein binding to Patched 1 protein (PTCH1) causes internalisation and destabilisation of this receptor. The smoothened receptor, which activity is inhibited by PTCH1, is then allowed to translocate to the plasma membrane to transduce the Hh signal[64]. Dysfunction of the Hh pathway in stem or precursor cells was found to contribute to tumorigenesis and neurodegenerative disorders[65, 66]. However, the mechanism by which SMOH activates Hh target genes in mammals remains unknown. Moreover, SMOH is present in very low concentrations in tissues, making overexpression in heterologous systems essential for biochemical and structural characterisation.

For this purpose, the human Hh signalling receptor SMOH was successfully overexpressed in its native conformational state in the yeast *S. cerevisiae*[67]. To achieve this, SMOH was subcloned in the expression vectors at the end of the N-terminus of the Multitag Affinity Purification (MAP) sequence, which was fused at the C-terminal end of SMOH. Yeast cells transformed with this vector were screened for expression of SMOH at the plasma membrane using antibodies directed against the hemagglutinin peptides present in the MAP sequence or C-terminal end of SMOH. Using a fluorescent derivative of a SMOH antagonist, the conformational state of SMOH expressed in yeast was visualized.

In a subsequent study, SMOH-MAP was solubilised with n-dodecyl-β-D-maltoside (DDM) detergent, and fully purified using a calmodulin and Ni-NTA affinity resin[68]. Additional replacement of a glycine by an arginine in the third intracellular loop of SMOH, and exchanging DDM with the fluorinated surfactant $C_8F_{17}TAC$ in the calmodulin resin increased the thermostability of SMOH.

Glucagon-like peptide-1 receptor

The glucagon-like peptide 1 (GLP-1) receptor belongs to the secretin receptor family of the GPCRs[3]. GLP-1 is the second incretin hormone discovered, after gastric inhibitory polypeptide (GIP)[69, 70]. GLP-1 is produced from posttranslational proteolytic cleavage of proglucagon, and is released from intestinal L-cells in response to nutrient ingestion. Binding of GLP-1 to the GLP-1 receptor on pancreatic β-cells leads to activation of adenylate cyclase activity, production of cyclic adenosine monophosphate (cAMP), and subsequent secretion of insulin from these cells. Diabetes type 2 patients display lowered GLP-1 concentrations and a reduced ability to promote insulin secretion[71]. Administration of GLP-1 peptide is able to induce glucose-dependent insulin secretion and regulate hyperglycaemia[72]. However, clinical effects appear to be dependent on the GLP-1 receptor response[73], which is not completely understood yet.

Yeast signalling assays were used to study individual ligand-receptor

G protein coupling preferences, and the quantification of the effect of GLP-1 receptor ligands on G protein selectivity[74]. Chimeric Gpa1/G_{α} transplants were created by replacing the five C-terminal amino acids of Gpa1 with the mammalian $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha z}$, and $G_{\alpha q}$ homolog sequences. The GLP-1 receptor was then integrated in these strains derived from *S. cerevisiae*, under the control of the pheromone-responsive FUS3 promoter. Results of this study revealed successful coupling of the GLP-1 receptor to chimeric transplants representing the human $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha q}$ G protein subunits. Additionally, responses to ligands of the GLP-1 receptor appeared to be influenced by the G protein subunit type, as exenatide significantly preferred GPL-1 receptor interaction with $G_{\alpha i}$. Exenatide is a GLP-1 mimetic and clinically approved therapeutic agent in the treatment of type 2 diabetes. Lastly, signalling properties of two small-molecule allosteric compounds were studied using the chimeric transplants, demonstrating the comprehensive possibilities of this system. These two compounds, compound 2 and BETP, activated the GLP-1 receptor when coupled to Gpa1/$G_{\alpha s}$. When the GLP-1 receptor was coupled to the Gpa1/$G_{\alpha i}$ chimera, compound 2 reduced receptor activity after receptor stimulation.

Recently, Shigemori and colleagues established a novel functional screening system for yeast-secreted peptides acting on the GLP-1 receptor[75]. This system integrated the yeast *S. cerevisiae* as host for the production of a peptide library and a functional detection system using Chinese hamster ovary (CHO) cells. The GLP-1 receptor expressed on CHO cells was successfully activated by various yeast-secreted GLP-1 receptor agonistic peptides. Especially, the yeast-secreted exendin-4 showed the highest activation of the GLP-1 receptor (10 times higher than yeast-secreted GLP1). This novel functional screening system can be formatted to discover novel peptides activating (other) druggable GPCRs.

Olfactory receptors (ORs)

Olfactory receptors (ORs) belong to the class A rhodopsin-like family of GPCRs[76] and ORs bind to odorant ligands. Minic et al. optimized a *S. cerevisiae* yeast system for functional expression of rat I7 OR and subsequent characterization. The engineered yeasts were lacking the endogenous Gpa1 subunit (the yeast

G_{α} subunit) and a mammalian G_{α} subunit (G_{olf}) was co-expressed. When the receptor was activated by its ligands, MAK kinase signalling was switched on and luciferase (as a functional reporter) synthesis was induced[77]. Marrakchi et al. successfully expressed human olfactory receptor OR17-40 in *S. cerevisiae* based on Minic's biosensor system to detect the conductometric changes[78]. Fukutani et al. improved the bioluminescence-based signalling assay system by deleting some genes to further improve the sensitivity of the firefly luciferase reporter assay and by replacing Gpa1p (yeast G_{α} subunit) with $G_{\alpha olf}$ (the olfactory-specific G_{α} subunit). This improvement successfully caused signal activation through both OR and yeast Ste2p[79]. The same team improved the biomimetic odor-sensing system by replacing the N-terminal region of mOR226 with the corresponding domain of the rat I7 receptor[80]. Likewise the assay sensitivity of some but not all ORs could be improved by the coexpression of odorant accessory binding proteins or the receptor transporting protein 1 short (RTP1S)[81].

Human serotonin 1A receptor

The human serotonin 1A receptor is also known as 5-hydroxytryptamine (5-HT) receptor 1A (HTR1A), which is a member of the 5-HT receptor subfamily[82]. Recently, Nakamura et al. constructed a yeast strain (IMFD-72ZsD) that expressed the human HTR1A[83]. They generated a fluorescent biosensor able to detect signalling by HTR1A in response to the neurotransmitter serotonin (5-HT). The biosensor signalling pathway used yeast cells harboring ZsGreen reporter genes (the tetrameric Zoanthus sp. green fluorescent protein) and a G_{i3}TP gene by replacing the endogenous GPA1 locus with a chimeric gene coding for a yeast Gpa1-human $G_{\alpha i3}$ protein. The improved ZsGreen-containing HTR1A-expressing strains displayed higher levels of ligand-dependent fluorescence than the previous EGFP-containing versions of HTR1A-expressing yeast strains. The authors further validated that this yeast biosensor strain allowed the characterization of an antagonist and the analysis of site-directed mutants of human HTR1A.

Other developments in *S. cerevisiae*

Resolution of 3D structures of target GPCRs provides good initial models for drug design. However, difficulties in expression and purification have been a major obstacle, as large quantities of high-quality pure protein are required for X-ray crystallography. Overexpression of GPCRs in a heterologous host is necessary for structural studies. Suitable variants, which improve expression and stability of receptors, must be selected.

Recently, a platform has been developed for rapid construction and evaluation of functional GPCR variants for structural studies, using *S. cerevisiae*[84]. GPCR variants were designed and the genes were generated as PCR fragments. The genes were integrated into the pDDGFP-2 plasmid, which contains a GAL1 promoter, by homologous recombination in *S. cerevisiae* strain FGY217 via introduction of a mixture of linearised plasmid and PCR products. Using an agar plate without uracil, the GPCR variant was selected. Functional expression was evaluated by radioligand binding assays, and monodispersity – as indicator for receptor purity – by fluorescence size exclusion chromatography. This platform enables a screening cycle within 6-7 days, and may be capable of identifying a suitable GPCR variant for crystallography.

Interestingly, insertion of T4 lysozyme (T4L) into the third intracellular (IL3) loops of GPCRs was suggested to stabilise the protein in a single conformation that would be more likely to crystallise than the unmodified protein, which contains a flexible IL3 loop and is structurally dynamic. Insertion of a small, readily crystallised, soluble protein into GPCRs that lack aqueous-facing protein-interacting surfaces may provide new protein-protein contacts for crystallisation.

However, modification of the IL3 loops in GPCRs could impede G protein activation, as these loops have been implicated in G protein coupling. Mathew and colleagues developed a random screening approach for receptor constructs allowing insertion of T4L into diverse positions in the IL3 loop of the yeast Ste2p receptor[85]. This allowed the identification of Ste2p-T4L fusion constructs which retained ligand binding properties and signalling functions.

Conclusions and future perspectives

The budding yeast *S. cerevisiae* provides a useful system for the study of human G protein-coupled receptors. Chimeric Gpa1/G_{α} G protein 'transplants' to improve coupling efficiency to human GPCRs are often used in yeast cells.

Two *S. cerevisiae* strains, IMFD-72 and IMFD-74, were engineered to express Gpa1/$G_{\alpha i3}$ and Gpa1/$G_{\alpha q}$ respectively, and to harbor additional alterations for improved readout of GPCR signalling. The IMFD-72 strain enabled improved cytometric separation to identify signal transduction mediated by the $G_{\alpha i}$-selective human somatostatin receptor 5, whereas the IMFD-74 strain improved sensing of neurotensin receptor type 1 signalling. These two strains should be applicable for screening of human $G_{\alpha i}$-specific or $G_{\alpha q}$-specific receptors of the GPCR family, and might be beneficial for the screening and development of novel ligands.

Constitutively active mutant (CAM) receptors could be used to discriminate inverse agonists from neutral antagonists. In this respect, inverse agonist T140-derived compounds were detected for the CXCR4 receptor. Using CAMs with varying levels of constitutive activity, three compounds were characterised as inverse agonists on the adenosine A_{2B} receptor, and their relative intrinsic efficacy was determined. In high-throughput screening of libraries CAMs could be used to discover inverse agonists for other GPCRs.

Functional G protein selectivity of a certain GPCR can be investigated when chimeric transplants representing the human G_{α} subunit subtypes are coupled to the endogenous GPCR signalling pathway in yeast. In the first study using yeast as a system to determine the relative efficacies of agonists and affinity values for both agonists and antagonists of the adenosine A_1 receptor, two agonists showed different efficacy when coupled to different G_{α} subtypes. Several amino acid residues at the C-terminus of G_{α} subunits were found to be essential in controlling adenosine A_{2B} receptor activation. The M_3 muscarinic acetylcholine receptor displayed functional selectivity too, as orthosteric antagonists were found to be inverse agonists when coupled to $G_{\alpha q}$, and low efficacy partial agonists when coupled to $G_{\alpha 12}$. This system was capable of identifying G protein selective allosteric modulators as well. Additionally,

responses to ligands of the glucagon-like peptide-1 receptor appeared to be influenced by the G protein subunit type. Exenatide, a clinically approved therapeutic agent in the treatment of type 2 diabetes, showed significant bias for the $G_{\alpha i}$ pathway. Extended application of this system might serve to improve selectivity and efficacy of therapeutic compounds for members of the GPCR family. More interestingly, the system would allow introduction of patient-specific mutations, whose effects on ligand signalling could be quantified, leading to efficient screening of personalized drugs.

In combination with mutagenesis, chimeric Gpa/G_{α} expressing yeast cells could provide a useful system for studying the role of certain amino acid residues in the structure of GPCRs in receptor activation.

The MMY24 yeast strain, which expresses chimeric Gpa1/$G_{\alpha i3}$, served as a useful model for investigating the role of distinct amino acids within the adenosine A_{2B} receptor. In combination with a random mutagenesis screen, residues $F71^{EL1}$ and $D74^{EL1}$ in the first extracellular loop were found to be involved in A_{2B} receptor activation. Site-directed and site-saturation mutagenesis on these residues revealed that a unique structural feature is essential for normal receptor activation. Random mutagenesis yielded three residues in transmembrane domain 4, second extracellular loop, and transmembrane domain 5 of the A_{2B} receptor that seemed to be involved in agonist potency and affinity. In the adenosine A_1 receptor, residues in the second extracellular loop in particular were found to be important for receptor activation through site-directed mutagenesis. Regarding somatostatin receptor 5, $N13^{N\text{-term}}$ and $N26^{N\text{-term}}$ appeared to be important in signalling, as revealed by site-directed mutagenesis. Also, random saturation mutagenesis showed the importance of a WXFG motif in the first extracellular loop in activation, but not ligand binding of complement factor 5a receptor. Additionally, site-directed mutagenesis revealed a novel role of EL3-N terminus cysteine pairs in GPCR activation and signalling. As the EL3-N terminus cysteine pairs and the WXFG motif in EL1 are both found in many GPCRs in the rhodopsin family, additional studies are warranted for a better understanding of the activation and signalling mechanism in these GPCRs.

Interestingly, site-directed mutation of $N295^{7.46}$ in the angiotensin II type

1 receptor yielded the development of a secretory expression system in which autocrine Ang II and Ang III could promote AGTR1-mediated signalling. This secretory expression system, which was able to detect high-affinity or highly-active agonists, could be promising for screening agonistic peptides by using plasmid libraries to express autocrine peptides with random or site-directed mutations.

The usefulness of a reporter gene system was emphasized in a number of studies. A highly sensitive method, which acts via a feedback signal activation mechanism, was developed for the detection of agonists of the human somatostatin receptor 5. This novel approach could advance yeast-based screening of agonistic ligands for a variety of other human GPCRs. The dual-color reporter system enabled simultaneous detection of dimerisation and signalling of SSTR5, and is expected to serve as a powerful tool for defining the mechanisms and functions of GPCR dimerisation. This would advance the development of new therapeutic agents.

The production of active human GPCRs in *S. cerevisiae* is limited by improper translocation of the receptor to the plasma membrane. Retention in the endoplasmatic reticulum is suggested to be the reason for many GPCRs to be improperly trafficked in the yeast system. Introduction of the yeast signalling sequence Ste2N to the N-terminus of human SSTR5 significantly improved agonist potency and improved receptor localisation to the plasma membrane.

Resolution of 3D structures of human GPCRs provides good initial models for drug design. For such structural studies, large quantities of high-quality pure protein are required. The X-ray crystal structure was solved for agonist-bound human M_2 muscarinic receptor, with or without allosteric modulator bound. Mimetic nanobodies for stabilisation of the protein were selected in *S. cerevisiae*. Human hedgehog signalling receptor, smoothened, was overexpressed, purified, and stabilised in yeast. High levels of functional, purified human adenosine A_{2A} receptor were achieved in yeast as well. These yields may help in X-ray crystallography, and provide structural knowledge of GPCRs. Interestingly, a platform was developed for rapid construction and evaluation of functional GPCR variants with improved expression and stability.

This GFP-based *S. cerevisiae* system enables a screening cycle within 6-7 days, and might be powerful in identifying a suitable variant for crystallography. In addition, insertion of T4 lysozyme into the third intracellular loop of the yeast Ste2p receptor resulted in stability, while retaining ligand binding properties and signalling functions. Insertion of such soluble proteins into the flexible loop of transmembrane proteins aids crystallisation of the protein, and might be applied to human GPCRs.

The considerable amount of new developments regarding human G protein-coupled receptors in a yeast platform has provided insight into GPCR activation and signalling, and facilitated agonist and antagonist identification. The budding yeast *S. cerevisiae* is expected to advance understanding of underlying mechanisms of GPCR activation and signalling, by providing a robust platform to perform structural and functional studies on. This would be of major importance in both improvement of selectivity and efficacy of existing therapeutic compounds, and in identifying potential drug targets.

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