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The good? The bad? The mutant! Characterization of cancer-related somatic mutations and identification of a selectivity hotspot in adenosine receptor

Wang, X.

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Chapter 2

G protein-coupled receptors expressed and studied in yeast. The adenosine receptor as prime example.

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Xuesong Wang, Gerard J.P. van Westen, Laura H. Heitman and Adriaan P. IJzerman
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Abstract

G protein-coupled receptors (GPCRs) are the largest class of membrane proteins with around 800 members in the human genome/proteome. Extracellular signals such as hormones and neurotransmitters regulate various biological processes via GPCRs, with GPCRs being the bodily target of 30–40% of current drugs on the market. Complete identification and understanding of GPCR functionality will provide opportunities for novel drug discovery. Yeast expresses three different endogenous GPCRs regulating pheromone and sugar sensing, with the pheromone pathway offering perspectives for the characterization of heterologous GPCR signaling. Moreover, yeast offers a ‘‘null” background for studies on mammalian GPCRs, including GPCR activation and signaling, ligand identification, and characterization of disease-related mutations. This review focuses on modifications of the yeast pheromone signaling pathway for functional GPCR studies, and on opportunities and usage of the yeast system as a platform for human GPCR studies. Finally, this review discusses in some further detail studies of adenosine receptors heterologously expressed in yeast.

Keywords: G protein-coupled receptor, engineered yeast system, adenosine receptors

Introduction

G protein-coupled receptors (GPCRs) are the largest family of membrane-bound proteins with approximately 800 members identified from the human genome[1,2]. They share a common basic architecture of seven-transmembrane (7TM) α-helices, linked by three intracellular (IL) and three extracellular (EL) loops, an extracellular amino terminus, and an intracellular carboxyl terminus[2]. According to their sequence homology, human GPCRs can be classified into five main families according to the GRAFS system: glutamate, rhodopsin, adhesion, frizzled/taste, and secretin[3]. Alternatively, GPCRs are divided in three main classes (A, B, and C)[4].

GPCRs respond to a wide diversity of extracellular endogenous ligands, including neurotransmitters and hormones. Intracellularly, GPCRs are coupled to different families of heterotrimeric G proteins, which contain three subunits, α, β, and γ[2]. Upon extracellular stimulation, conformational changes in GPCRs leads to the replacement of GDP for GTP at the G_{α} subunit resulting in the dissociation of the $G_{\beta\gamma}$ subunit from G_{α} and further interactions with effector proteins in the cell[5,6]. Based on sequence similarity and functionality, the G_{α}-subunit family is divided into four major subfamilies, $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$[7,8].

GPCRs play a crucial role in human physiology due to their abundant distribution and numerous GPCR-related downstream pathways. Moreover, they are substantially involved in human pathophysiology[6]. During the past decades, GPCRs have thus been investigated as pharmacological targets with the focus on their extracellular ligand binding site[9]. The major disease indications for GPCR modulators have shifted over the years from high blood pressure to metabolic diseases, as well as several central nervous system disorders, and more recently also to tumor initiation and progression[6–8,10–12]. Their role in both physiological and pathophysiological conditions have made GPCRs the target of approximately 30% of therapeutic drugs to date[9]. Thereby, ongoing further characterization of GPCR functionality will offer new opportunities for drug discovery. However, due to the complexity of mammalian GPCR signaling pathways, using mammalian cells as the host for investigating GPCR signaling is relatively time-consuming and can result in ambiguous output. The latter can be problematic due to the wide distribution and variety of endogenous receptors and their ligands in such cells, and also expensive. In this case, engineered yeast systems provide a relatively cost-effective and useful model system to analyze human GPCRs.

In this review, we will discuss strategies to link human GPCR expression and functionality to the endogenous yeast pheromone mating pathway in *Saccharomyces cerevisiae* (*S. cerevisiae*) as well as expression strategies in *Pichia Pastoris* (*P. pastoris*). Finally, we will focus on functional studies on adenosine receptor signaling using yeast reporter systems.

General features of yeast

Among the many yeast species *S. cerevisiae* and *P. pastoris* have been genetically well characterized as a model system. The first crystal structures of recombinant mammalian membrane proteins were obtained by their overexpression in these two yeast species[13,14]. Since then, yeast expression has been frequently used for harvesting, purifying and subsequently obtaining crystal structures of membrane proteins deposited in the ProteinDataBank[15].

P. pastoris, as a recombinant expression host system, is an engineered methylotrophic microorganism using methanol as carbon and energy source[16]. The strain Y-11430 (wild-type) is not used for heterologous protein expression due to low transformation efficiency, while the GS115 strain is one of the most commonly used expression systems particularly for industry[17]. The *P. pastoris* system shows high similarity to advanced eukaryotic expression systems like CHO and HEK293 cell lines, as cotranslational and posttranslational modifications also occur[18]. However, this inexpensive yeast system constitutes a more rapid expression platform, and *P. pastoris* does not overglycosylate therapeutic proteins in comparison to *S. cerevisiae*[18].

The budding yeast *S. cerevisiae* expresses three different endogenous GPCRs involved in sugar and pheromone sensing[19]. Glucose sensing is mediated via the yeast G protein-coupled receptor-1 (Gpr1) and the G_{α} protein Gpa2 (Figure 1A)[20,21], and pheromone sensing via GPCRs α-factor receptor (Ste2) and a-factor receptor (Ste3), as well as the G_{α} protein Gpa1 (Figure 1B)[22]. During the past decades the yeast pheromone pathway has been the most extensively studied GPCR signaling cascade[23,24]. Similar to mammalian GPCR signaling, Ste2 or Ste3, couples to the yeast trimeric G protein upon activation by a- or α-factor pheromones, consisting of Gpa1(α subunit), Ste4 (β subunit) and Ste18 (γ subunit)[19,25]. Activation of the receptor results in the dissociation of the βɣ-dimer from the α-subunit. The βɣ-dimer further couples and induces mating-specific responses by activating the mitogen-activated protein kinase (MAPK) signaling cascade[26]. Ultimately, the translocation of the transcription factor Ste12 mediated by activation of the MAPK cascade further regulates the expression of numerous mating pathway target promotors[27–29]. Based on the similarity between the yeast mating pathway and mammalian GPCR signaling pathways, human GPCRs have been expressed and further coupled to a reporter gene output in order to more broadly study GPCR signaling[23].

Modifications in engineered yeast system

Yeast has been used as a vehicle for more than three decades for the structural and functional characterization of endogenously and heterologously expressed GPCRs[23,30,31], for the discovery of novel GPCR ligands (deorphanization)[32,33], for metabolic engineering purposes[34,35], and for the minimization of GPCR signaling

complexity[36]. With the deletion of yeast GPCRs, the yeast system provides a synthetic 'null' GPCR background for investigating non-native receptors[23,25,37]. In comparison to mammalian systems, shorter doubling time and simple cell culture are among the benefits when studying GPCRs in yeast[38]. The yeast mating pathway with its resemblance to mammalian GPCR signaling pathways, therefore, offers a framework with multiple engineering possibilities to link heterologous GPCRs to a reporter output[36]. Hereby, we will discuss the modifications of the natural yeast pathway to investigate GPCR signaling and deorphanization (Figure 1C).

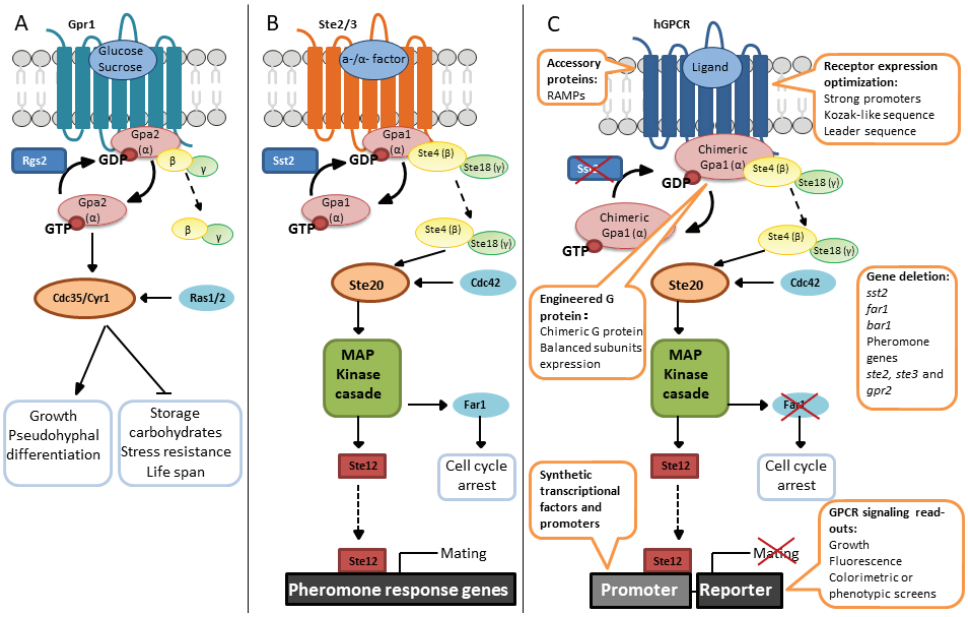

Figure 1. Overview of GPCR signaling pathways in *S. cerevisiae*, adapted from Versele *et al.* (2001)[19] and Lengger *et al.* (2020)[63]. A) Glucose signaling via Gpr1, B) pheromone signaling via Ste2 and Ste 3 and C) modifications of pheromone signaling pathway for coupling to human GPCRs.

Engineered G proteins

In general, heterologous GPCRs show preferences in G protein coupling depending on their native G_{α} coupling[39]. Although it has been reported that heterologous GPCRs can couple to the endogenous Gpa1 subunit[34], higher coupling efficiency has been achieved by using chimeric G_{α} subunits, which are thus commonly preferred[37,40]. In the chimeric G_{α} subunits, the last five amino acids of the C-terminus have been transplanted based on the mammalian G_{α} sequence to improve receptor recognition[41,42]. Apart from identifying matching GPCRs, optimization of G protein subunit expression is crucial for successfully engineering and restoring GPCR signaling. It has been computationally and experimentally confirmed that optimally balanced levels of G protein subunits maintain high pathway output but low basal activity[36,43,44].

Gene deletions

The yeast pheromone pathway regulates mating initiation[19], however, there is no requirement of mating genes for studying GPCR signaling. Instead, they may even intervene with functional studies of heterologous GPCRs. In order to boost GPCR signaling strength, certain pheromone pathway-related genes were thus eliminated, for example through knock-out of the three native GPCRs, down regulation of Gpa1 and SST2 expression, as well as deletion of the FAR1 gene, a cell cycle arrest inducer during mating[34,38,40,45,46]. More recently, deletion of BAR1 and pheromone genes has been proven to (further) minimize the pheromone response[32]. Moreover, yeast proteases may target intracellular loops of GPCRs resulting in receptor degradation. Thus, deletion of the central portion of the intracellular loops of heterologous GPCRs and the usage of a protease-deficient yeast strain have been shown to increase receptor amounts[47–49]. In addition, targeted insertion of defined sequences at the deletion sites allows re-introduction of key signaling genes, which provides a highly tunable GPCR signaling pathway in the yeast system[36].

Optimization of receptor expression

Multiple GPCRs have been successfully expressed in yeast cells, while the development of expressing functional GPCRs is still ongoing. In *P. pastoris*, GPCRs are typically and deliberately truncated at the C-terminus to prevent degradation, which increases the expression and stability of receptor[16,50]. Besides, strong constitutive promoters, such as TDH3 or PGK1, have also been reported to increase GPCR levels[32,51]. Additionally, agonist-mediated fluorescence reporter intensity could be dramatically increased by the upstream insertion of the Kozak-like sequence (-AAAAAAAUGUCU-) to a neurotensin GPCR open reading frame[51,52].

To increase GPCR expression by expanding the post-translational processing, the fusion of a leader sequence to the N-terminus of the receptor has been shown to assist the plasma membrane insertion of the receptor[53]. An early study on the β_2-adrenoreceptor suggested that replacing part of the receptor N-terminus with the corresponding sequence of Ste2 supported the expression and functionalization of the β_2-adrenoreceptor[54]. Moreover, the addition of a hydrophobic pre-prosequence resulted in higher expression and better insertion into the membrane for 12 different human GPCRs in a GPCR fusion study[55]. Although all these 12 GPCRs were successfully expressed with high yields, all except the adenosine A_{2A} receptor were primarily observed within the cells and detected with intact or partially cleaved leader sequences, indicating the problem of improper ER translocation and thus the limiting step of human GPCR production in *S. cerevisiae*[55].

Accessory proteins

Despite the transcriptional and post-transcriptional controls discussed above, lack of accessory proteins also hampers the development of functional GPCR assays in yeast[40,55,56]. Co-expression of human odorant receptor OR7D4 with an accessory

protein, receptor-transporting protein, have been reported to increase receptor transportation to the yeast membrane[57]. Other accessory proteins, such as receptor-activity modifying proteins (RAMPs), can dimerize with GPCRs and modulate their activity, including ligand selectivity, transport to the cell surface, internalization and even downstream signaling[58].

Synthetic transcriptional factors and promoters

MAPK cascade-mediated Ste12 translocation regulates the expression of endogenous mating pathway target promoters in yeast cells[29]. Thus, the use of Ste12 as a controller for reporter gene expression via the FUS1, FUS2 and FIG1 promoters is the most commonly used design for studying GPCR signaling in yeast[59–62]. However, the expression strength of Ste12 needs to be limited to prevent impaired cell growth[36]. Therefore, in this case, coupling heterologous GPCR signaling to a synthetic transcription factor is able to target the reporter gene without influencing yeast mating target promoters[63]. For instance, compared to the FIG1 promoter, using the synthetic promoter PLexA(4x) results in a 7-fold increase of green fluorescent protein (GFP) expression upon the activation of olfactory OR1G1 GPCR in response to decanoic acid[34].

GPCR signaling read-outs

Functional GPCR screening assays often involve cell growth, fluorescence, and/or colorimetric or phenotypic screens[36,64,65]. A growth assay coupling GPCRs to a HIS3 reporter gene was designed for ligand screening of inverse agonists and weak partial agonists[66,67]. Complementarily, inverted reporter systems coupling to CAN1 and FUI, encoding permeases, can only survive with the addition of agonists and have been used to investigate non-functional GPCRs, such as mutant GPCRs with abolished receptor activation[67–69]. Moreover, GPCR antagonists have been investigated using the inverted reporter system with fluorescent read-outs[70]. Overall, the usage and optimization of novel sensitive fluorescent markers with high signal-to-noise ratio have become the trend for functional assays of GPCR signaling in yeast[51,71,72].

GPCR studies in yeast

As mentioned above, *P. pastoris* is a preferred platform for GPCR production due to its high expression capacity[31]. Functional characterization of GPCR signaling has been extensively studied employing the *S. cerevisiae* mating pathway[23,25,37]. In this section, we will discuss some examples in which yeast systems are used as a crucial platform in the development of GPCR purification and signaling characterization, as well as for GPCR deorphanization studies (Table 1).

Purification of GPCRs

Crystal structures of GPCRs in complex with various ligands and/or G proteins

nowadays provide numerous initial models for drug design and discovery[6]. However, large quantities of high-quality pure protein are always required for X-ray crystallography, which constitutes a major hurdle in GPCR expression and purification. Therefore, relatively cheap and easy-to-handle yeast systems are used as expression systems for GPCRs, also to improve expression and stability of the receptors[31,73].

S. cerevisiae has been developed as a chassis for rapid expression and characterization of four functional human GPCRs and their variants as a starting point for X-ray crystallography, viz. β_2-adrenoreceptor, acetylcholine M_2 receptor, histamine H_1 receptor and neurotensin NTS_1 receptor[73]. In this study, the expression of a stabilized H_1 receptor variant was scaled up to 65 pmol/mg in *P. pastoris* and successfully purified for crystallization trials, indicating that the combined strategy of using *S. cerevisiae* for rapid screening and *P. pastoris* for high expression could be effective for GPCR structural studies[73]. Human smoothened receptor with an N-terminal purification tag has been successfully expressed, visualized, and purified from the *S. cerevisiae* system[74]. High expression levels of N-terminal histidine-tagged β_2-adrenoreceptor were successfully achieved in *P. pastoris* with optimized codon usage and further purified with hydroxyapatite and gel-filtration columns[75]. In fact the *P. pastoris* expression system has been specifically used to produce membrane proteins, such as calcium and potassium channels, nitrate and phosphate transporters, and the H_1 histamine receptor[31]. The fusion of T4 lysozyme (T4L) into the third intracellular loop of GPCR favors GPCR stabilization and crystallization[76]. *P. pastoris* was also used to express the H_1 receptor-T4L fusion protein, which was later used for a crystallographic study of the receptor in complex with doxepin, a first-generation H_1 receptor antagonist[77,78]. Furthermore, functional cannabinoid receptors (both CB_1 and CB_2 receptors) with a c-myc epitope and a hexahistidine tag at the C-terminus were successfully expressed and purified in the *P. pastoris* system[79,80]. However, non-homogenous glycosylation and the presence of unprocessed α-factor sequence were detected at the N-terminus of the CB_2 receptor expressed in the same *P. pastoris* system, where this unprocessed α-factor appeared to be the cause of poor ligand binding at the CB_2 receptor[81]. Hence, it makes yeast suitable for CB_2 receptor purification, but less or unsuitable for functional characterization of the protein.

Characterization of novel ligands and GPCR signaling

The human β_2-adrenoreceptor was the first functional heterologously expressed GPCR in yeast responding to its agonist isoproterenol[54]. Since then, many more human GPCRs have been linked to the yeast pheromone pathway for functional studies[24]. Most sub-families of class A and few receptors of class B GPCRs have shown successful expression in yeast[34,37,38,47,54,66,82–93].

A comparative study between the *S. cerevisiae* and a mammalian system with respect to the $P2Y_1$ receptor indicated that the reporter gene HIS3-coupled yeast system

was suitable for screening both agonists and antagonists of the $P2Y_1$ receptor[94]. Wild-type and the constitutively active mutant (N119S) CXCR4 chemokine receptor were expressed in *S. cerevisiae* coupled to the FUS1 promoter controlling reporter genes HIS3, FUI, and lacZ, and tested for receptor signaling mediated by T140 derivatives[67]. Of note, relatively high concentrations of chemokine were needed to obtain a response in the yeast system, as compared to more conventional mammalian functional assays. Besides, novel allosteric CXCR4 antagonists were identified from a screening a library of 160,000 known chemokine receptor ligands using the *S. cerevisiae* system coupled to reporter genes HIS3 and lacZ[95]. Propionate and further short-chain carboxylic acids were identified as agonists on orphan receptors GPR41 and GPR43 from routine ligand bank screening using the yeast system coupled to β-galactosidase activity[96]. Similarly, yeast-based screening assays on GPR68 suggested the benzodiazepine lorazepam as a non-selective agonist of this orphan receptor[85]. For GPR119 a novel agonist PSN375963 was identified with a similar potency to the reported endogenous ligand, oleoylethanolamide[97–99]. The usage of yeast systems with different G protein modifications for glucagon-like peptide-1 receptor revealed the importance of the co-expression of receptor activity-modifying protein-2 (RAMP-2) with the receptor in ligand binding and G protein selectivity experiments[56].

The lacZ reporter gene was used as functional read-out of acetylcholine M_3 receptor ligands in *S. cerevisiae* strains containing different chimeric G proteins, indicating functional selectivity of this receptor upon coupling to different G_α subunits[100]. The same reporter gene was coupled to $P2Y_{12}$ receptors expressed in *S. cerevisiae* as a functional read-out of agonist-induced activation, revealing similar functional pharmacological properties between human and murine $P2Y_{12}$ receptors[101]. Functional chimeras of $P2Y_1$, $P2Y_2$ and/or leukotriene BLT_1 receptors have been successfully expressed in an *S. cerevisiae* system with the lacZ reporter gene to define regions required for ligand-induced activation. This provided a new approach to study receptors with low coupling efficiency in the given system[102]. A mutagenesis study of complement peptide $C5a_1$ receptor has been done in an *S. cerevisiae* system with the HIS3 reporter gene, demonstrating a particular role of the WXFG motif in the first extracellular loop during $C5a_1$ receptor activation[103]. In order to characterize antagonists and analyze mutations of 5-hydroxytryptamine receptor 1A, a high-sensitivity yeast system was developed with an engineered G_α subunit and coupled to a fluorescent reporter, ZsGreen[104]. Additionally, yeast strains coupling human GPCR activation to the HIS3 reporter gene were used in a mutagenesis study for the investigation of the role of the C-terminus in G protein activation by the human hydroxycarboxylic acid receptors 2 and 3[86]. Recently, light-sensing human rhodopsin has been coupled to the yeast mating pathway with successful expression and characterization of disease-causing mutations, enabling cost-efficient ligand screening in a semi-high-throughput format[38,105].

Biosensors

For the investigation of the neurotensin NTS_1 receptor, a fluorescence-based microbial yeast biosensor has been constructed to monitor receptor activation stimulated by agonists, which is also a promising approach in the diagnosis of NTS_1 receptor-related diseases and agonist development[106]. For the angiotensin AT_1 receptor, a fluorescence-based yeast biosensor was also developed with the yeast-human chimeric G_α for the introduction of single mutations into the receptor to screen agonistic peptides[107]. In this system, the engineered yeast cells produced and secreted the autocrine Ang II peptide and an analog, which activated AT_1 receptors expressed in the same system[107]. Engineered human $P2Y_{14}$ receptors with different ligand specificities and efficacies expressed in *S. cerevisiae* in combination with a fluorescent read-out were suitable biosensors for detecting ligands in complex mixtures, and for differentiating among (stereo)chemically related ligands[108]. Moreover, an olfactory biosensor has been developed in engineered *S. cerevisiae* yeast cells expressing human olfactory receptor OR17-40 to detect odorants with a high sensitivity and selectivity[109].

Deorphanization

In an early deorphanization study in yeast, the olfactory receptor KIAA0001, now known as $P2Y_{14}$ receptor, was expressed and coupled to different G_α subunits, which ultimately identified UDP-glucose as a specific agonist[82]. Human receptor OSGPR1116, now known as GPR119, has been identified with fatty-acid ethanolamides as agonists in a yeast system[97]. Recently, seven human orphan olfactory receptors were expressed in a yeast system, their presence determined by immunofluorescence microscopy, and eventually screened with 57 chemicals, suggesting the value of yeast-based screening systems for olfactory receptor deorphanization[33].

Adenosine receptor studies in yeast

The adenosine receptors belong to Class A, rhodopsin-like GPCRs and exist of four subtypes, A_1, A_{2A}, A_{2B} and A_3. They have attracted much attention in recent years as therapeutic targets[3]. Depending on the adenosine receptor subtype, binding of extracellular adenosine leads to activation of different downstream signaling cascades[110–115]. The A_1 and A_3 receptors inhibit adenylate cyclase and reduce cAMP levels mainly via G_i-coupling[112,113]. Conversely, A_{2A} and A_{2B} receptors are mainly coupled to G_S proteins and increase the levels of cAMP[114,115]. The A_1 receptor, abundant in the central nervous system (CNS) and identified in numerous peripheral tissues, plays a pivotal role in neuronal, renal and cardiac processes[116–119]. Thus, the A_1 receptor has been under investigation as a drug target for brain pathologies, such as pain, depression and memory disorders[120–123]. High expression levels of A_{2A} receptor are found in the CNS and the immune system[124]. The A_{2A} receptor is therefore involved in CNS disorders,

Table1. Examples of human GPCR studies in yeast systems.

Receptors	Yeast species	Applications	Read-outs	Reference
5-hydroxytryptamine receptor 1A	*S. cerevisiae*	Characterization of ligand and site-directed mutants	Fluorescence (ZsGreen)	[104]
Acetylcholine M_2 receptor	*S. cerevisiae*	Crystallization	-	[73]
Acetylcholine M_3 receptor	*S. cerevisiae*	Functional selectivity	Fluorescence (β-galactosidase)	[100]
Adenosine A_1 receptor	*S. cerevisiae*	Ligand characterization	Fluorescence (β-galactosidase)	[134]
	S. cerevisiae	Structural characterization	Growth (HIS3)	[135]
Adenosine A_{2A} receptor	*P. pastoris*	Crystallization	-	[146]
	P. pastoris	Purification	-	[147–149]
	S. cerevisiae	Expression purpose	Radioligand binding; fluorescence (GFP)	[55,142–145]
	S. cerevisiae	Thermostabilizing mutation screening	Growth (HIS3); fluorescence (β-galactosidase)	[136]
Adenosine A_{2B} receptor	*S. cerevisiae*	Structural characterization	Growth (HIS3)	[137–141]
	S. cerevisiae	Characterization of cancer-related mutations	Growth (HIS3)	[151]
Adenosine A_3 receptor	*S. cerevisiae*	Expression purpose	Fluorescence (mCitrine)	[150]
Angiotensin AT_1 receptor	*S. cerevisiae*	Microbial biosensor	Fluorescence (GFP)	[107]
Cannabinoid CB_1 receptor	*P. pastoris*	Purification	-	[80]
Cannabinoid CB_2 receptor	*P. pastoris*	Purification	-	[79,81]
Complement peptide $C5a_1$ receptor	*S. cerevisiae*	Structural characterization	Growth (HIS3)	[103]
CXCR4	*S. cerevisiae*	Ligand screening	Growth (HIS3 and FUI); fluorescence (β-galactosidase)	[67,95]
Glucagon-like peptide-1 receptor	*S. cerevisiae*	Ligand screening and G protein selectivity	Fluorescence (β-galactosidase)	[56]
GPR119	*S. cerevisiae*	Ligand screening and deorphanization	Fluorescence (β-galactosidase)	[97–99]
GPR41 and GPR43	*S. cerevisiae*	Ligand screening	Fluorescence (β-galactosidase)	[96]
GPR68	*S. cerevisiae*	Ligand screening	Growth (HIS3)	[84]
Histamine H_1 receptor	*P. pastoris*	Crystallization (fused with T4L)	-	[76-78]
	S. cerevisiae	Crystallization	-	[73]
Hydroxycarboxylic acid receptors 2 and 3	*S. cerevisiae*	Structural characterization	Growth (HIS3)	[86]
Leukotriene BLT_1 receptor	*S. cerevisiae*	Functional characterization of receptor chimeras	Fluorescence (β-galactosidase)	[102]
Neurotensin NTS_1 receptor	*S. cerevisiae*	Purification	-	[73]
	S. cerevisiae	Microbial biosensor	Fluorescence (GFP)	[106]
Olfactory receptor	*S. cerevisiae*	Microbial biosensor	Conductance	[109]
Olfactory receptor 10S1	*S. cerevisiae*	Deorphanization	Fluorescence (GFP)	[33]
Olfactory receptor 2T4	*S. cerevisiae*	Deorphanization	Fluorescence (GFP)	[33]
$P2Y_1$ receptor	*S. cerevisiae*	Ligand screening	Growth (HIS3)	[94]
$P2Y_1$ and $P2Y_2$ receptors	*S. cerevisiae*	Functional characterization of receptor chimeras	Fluorescence (β-galactosidase)	[102]
$P2Y_{12}$ receptor	*S. cerevisiae*	Characterization of murine receptor in comparison to human receptor	Fluorescence (β-galactosidase)	[101]
$P2Y_{14}$ receptor	*S. cerevisiae*	Deorphanization	Fluoresence (β-galactosidase)	[82]
	S. cerevisiae	Biosensor	Fluoresence (β-galactosidase)	[108]
Rhodopsin	*S. cerevisiae*	Functional characterization of disease-causing mutations	Fluorescence (GFP, mCherry)	[38,105]
Smoothened receptor	*S. cerevisiae*	Purification	-	[74]
β_2-adrenoreceptor	*P. pastoris*	Purification	-	[73,75]
β_2-adrenoreceptor	*S. cerevisiae*	Expression and functional characterization of the wild-type receptor	Radioligand binding; fluorescence (β-galactosidase)	[54]

inflammation, pain and drug addiction[116,125,126]. The A_{2B} receptor is ubiquitously expressed in many organs, as well as on microvascular, endothelial and immune cells[127,128]. This receptor is only activated by high concentrations of adenosine and is known to modulate inflammation and immune responses in several pathological conditions, such as cancer, diabetes and lung diseases[129,130]. The A_3 receptor is suggested to mediate allergic responses, airway contraction and apoptotic events in certain cell types[117,118]. High expression levels of A_3 receptor have been determined in tumor cells compared to healthy cells, demonstrating its potential role as a tumor marker[131]. In the tumor microenvironment adenosine accumulation is mediated via the catabolism of extracellular ATP to adenosine by CD38, CD39, and CD73, which suppresses anti-tumor immune responses via the activation of adenosine receptors[132]. Therefore, multiple antagonistic antibodies and small molecule inhibitors targeting adenosine receptors have been developed as new strategies in cancer immunotherapy and display therapeutic efficacy in clinical trials against different solid tumors[133]. During the past years, it has become clear that activation of adenosine receptors not only depends on the ligand binding and G protein coupling sites, but also on other, more distant regions in the receptor architecture[134–141]. Yeast systems, in this case, have been used as a host for adenosine receptors for receptor purification and the characterization of ligands, receptor structure and function, and disease-related mutations (Table 1).

Expression and purification of adenosine receptors

The first functional human adenosine receptor expressed in a yeast system was the A_{2A} receptor using *S. cerevisiae* and confirmed by a radioligand binding assay[142]. In this study, the expression and functionality of A_{2A} receptors were not altered by the co-overexpression of several ER-resident proteins, suggesting that interactions with these proteins did not decrease human GPCR expression in yeast[142]. Later on, the A_{2A} receptor with a C-terminal GFP tag was expressed and analyzed in *S. cerevisiae*, and the obtained results suggested that limited heterologous GPCR expression was caused by translational or post-translational events[143]. The same team also obtained and selected a yeast strain with a high expression level using flow cytometry, which was eventually used to purify the A_{2A} receptor[144]. Further optimizations were performed by fusing a purification tag to the A_{2A} receptor, as well as developing a suitable purification scheme, resulting in large enough quantities for spectroscopic characterization[145]. Furthermore, in order to better understand the improper trafficking and inactivation of GPCRs in heterologous expression systems, 12 human GPCRs with a GFP tag were transformed in *S. cerevisiae*[55]. Among these GPCRs, only the A_{2A} receptor proved active and was located primarily at the plasma membrane with proper leader sequence processing, indicating a crucial role of translocation in producing active human GPCRs in *S. cerevisiae*[55]. A crystal structure of the A_{2A} receptor with the complete third intracellular loop and an allosteric inverse-agonist antibody was obtained using *P. pastoris* as the expression host of the receptor[146]. Moreover, the A_{2A} receptor was expressed in *P. pastoris* and encapsulated into

styrene maleic acid lipid particles (SMALPs) to increase thermostability, which enabled purification procedures without the requirement of detergent[147]. The same combination of *P. pastoris*-expressed human A_{2A} receptor and SMALPs has recently been used to characterize the binding capability[148], and to investigate ligand-induced conformational changes of the A_{2A} receptor in response to an inverse agonist and full agonist[149].

More recently, the A_3 receptor was successfully expressed in *S. cerevisiae* in which the C-terminus was replaced by the corresponding tail of the A_{2A} receptor[150]. The A_3/A_{2A} chimeric receptor significantly increased receptor expression and decreased unfolded protein in comparison to wild-type A_3 receptor. Thus, chimeric receptor variants can be used as a strategy to produce "difficult-to-express" receptors in yeast for further drug discovery[150].

Functional characterization of adenosine receptors

In order to characterize both antagonists and agonists of the A_1 receptor, *S. cerevisiae* strains expressing the receptor and different human-yeast chimeric G proteins were used in combination with a lacZ reporter gene[134]. In this study, β-galactosidase activity was measured as a read-out of receptor activation, suggesting that R-PIA was an agonist with high efficacy when coupling to $G_{\alpha o}$, $G_{\alpha i1/2}$, and $G_{\alpha i3}$ proteins, while VCP-189 was an agonist with low efficacy selectively coupling to the $G_{\alpha i1/2}$, and $G_{\alpha i3}$ proteins[134]. Besides, results obtained in a mammalian system were in general agreement with those in the yeast system, except for VCP-189 which also activated $G_{\alpha o}$ coupling in mammalian cells[134]. The role of extracellular loops in receptor activation and allosteric modulation was examined in another study in which the adenosine A_1 receptor was expressed in yeast[135]. Here, receptor signaling was coupled to yeast growth via a chimeric Gpa1/$G_{\alpha i3}$ protein, and single alanine mutations were introduced to the extracellular loops of the receptor via site-directed mutagenesis. Results obtained from this study implicated the importance of many residues located at the second and third extracellular loops in receptor activation, and identified two residues, $W156^{EL2}$ and $E164^{EL2}$, regulating the effect of the allosteric modulator[135].

Screening of thermostabilizing mutations in the adenosine A_{2A} receptor was performed in a yeast system with an engineered G protein and HIS3 and lacZ reporter genes[136]. Alanine mutation of residues R199 and L208 completely abolished the constitutive activity of the A_{2A} receptor. Besides, decreased potency was observed on mutant receptor R199A while reduced efficacy was displayed on mutant receptor L208A, supporting key roles of these residues in receptor signaling[136].

Several mutagenesis studies have been performed on the A_{2B} receptor in a yeast system with engineered G protein and HIS3 reporter gene[137–141]. Inverse agonists of the A_{2B} receptor were discovered using constitutively active mutants (CAMs) expressed in the yeast system[139]. In this study, CAMs with different constitutive activity levels were used to determine the relative intrinsic efficacy of the three inverse agonists,

DPCPX, MRS1706, and ZM241385[139]. Two high-level CAMs were identified to lock the receptor in the active state and to be irresponsive to these inverse agonists[139]. In a study focusing on the interaction between the A_{2B} receptor and the C-terminus of G_{α} subunits, wild-type and mutant receptors were investigated in eight yeast strains expressing different chimeric G proteins[137]. Three residues, R103, I107 and L236, were revealed to be important in receptor activation via altering G protein interaction and activation[137]. Besides, key residues in the NPxxY$(x)_{5,6}$F motif and helix 8 of the receptor were screened in the yeast system with chimeric Gpa1/$G_{\alpha i3}$ protein[138]. Four mutants P287A, Y290A, R293A and I304A were identified with a complete loss of function, and eight more residues, N286, V289, Y292, N294, F297, R298, H302 and R307, were also found to be vital in receptor activation[138]. A random mutagenesis study on the first extracellular loop of the adenosine A_{2B} receptor expressed in an *S. cerevisiae* strain demonstrated the necessity of a polar residue at position 74[140]. Various mutations at residues 71 and 74 were able to dramatically influence receptor activation, suggesting that the first extracellular loop of A_{2B} receptor is (also) essential for receptor activation (Figure 2)[140]. Furthermore, random mutagenesis on the fragment encoding the second extracellular loop flanked by the fourth and

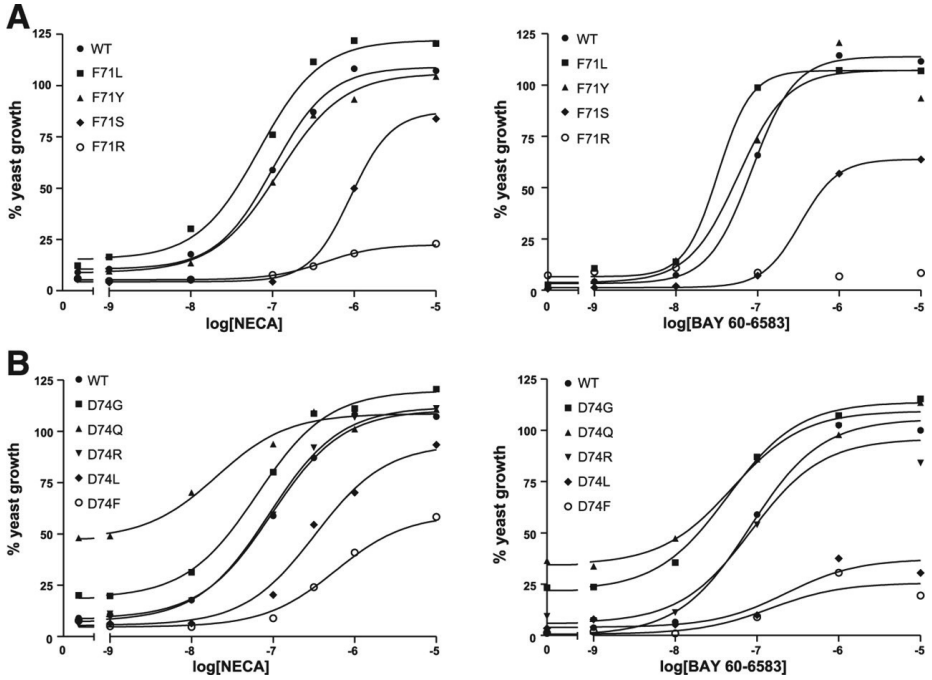

Figure 2. Representative concentration-growth curves of wild-type and A) mutant adenosine A_{2B} receptors of residue F71 and B) mutant A_{2B} receptors of residue D74 in response to the ribose agonist NECA or the non-ribose agonist BAY 60-6583. The maximum activation level of wild-type A_{2B} receptors was set at 100%, the background of the selection medium was set at 0%. Mutations are shown in the numbering of the A_{2B} receptors amino acid sequencing. WT in the figures represents wild-type. Reproduced with permission from Peeters *et al*. (2011)[140].

fifth transmembrane helices resulted in 22 different single and double mutant receptors with decreased constitutive activity and agonist potency[141]. Comparing these constitutively inactive mutants (CIMs) and CAMs previously identified from the same fragment, six residues were found in both CAM and CIM screening, indicating their crucial roles in both activation and inactivation of the A_{2B} receptor (Figure 3)[141]. Recently, the same yeast strain was used in characterizing cancer-related somatic mutations in the A_{2B} receptor[151], as described in **Chapter 4**. These mutations might even be cancer-specific as they did not match any point mutations identified from the natural variance set[151]. Several of these cancer-related mutations caused significantly altered receptor pharmacology (Figure 4)[151].

Conclusion

A considerable number of human GPCRs has been investigated in a yeast platform with different purposes, including protein purification, investigation of receptor activation and signaling, as well as ligand identification. *P. pastoris* yeast strains can be highly efficient and cost-effective expression systems for GPCRs of interest for the purpose of protein purification and eventually crystallization/ structure elucidation. The pheromone signaling pathway of the budding yeast *S. cerevisiae* has been engineered with various modifications to provide a robust platform for functional studies on human GPCRs, both wild-type and mutated. Therefore, these yeast platforms are a very useful and attractive addition to the more commonly employed mammalian cell lines for receptor expression, such as CHO and HEK293 cells.

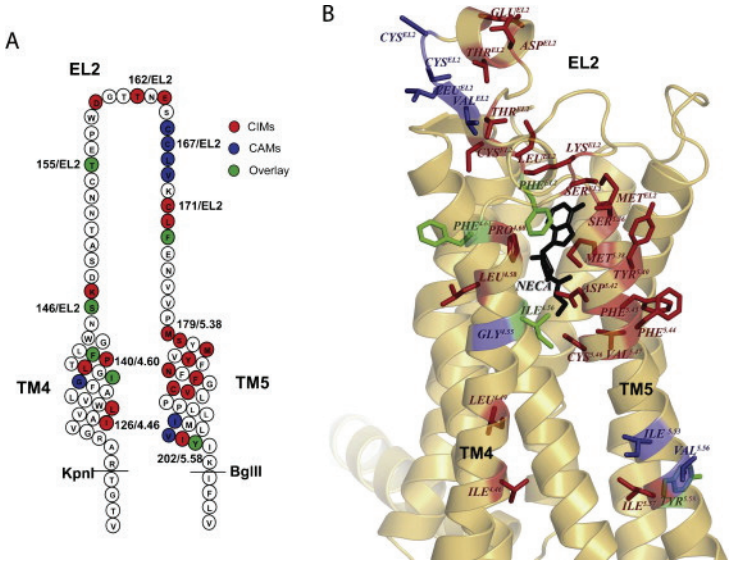

Figure 3. Location of constitutively inactive mutants (CIMs) and constitutively active mutants (CAMs) in the human adenosine A_{2B} receptor. (A) A snake-plot structure of the fragment used in the CIMs and CAMs screening. The positions are shown in the numbering of the A_{2B} receptors amino acid sequencing as well as according to the Ballesteros–Weinstein numbering[152]. (B) Based on the multiple sequence alignment, the mutated residues identified from the screen were mapped onto the crystal structure of the A_{2A} receptor (PDB: 3YDV). The positions are labeled according to the Ballesteros–Weinstein numbering[152]. The CIMs are shown in red, CAMs in blue, and positions identified in both screens are shown in green (overlay). Reproduced with permission from Peeters *et al*. (2014)[141].

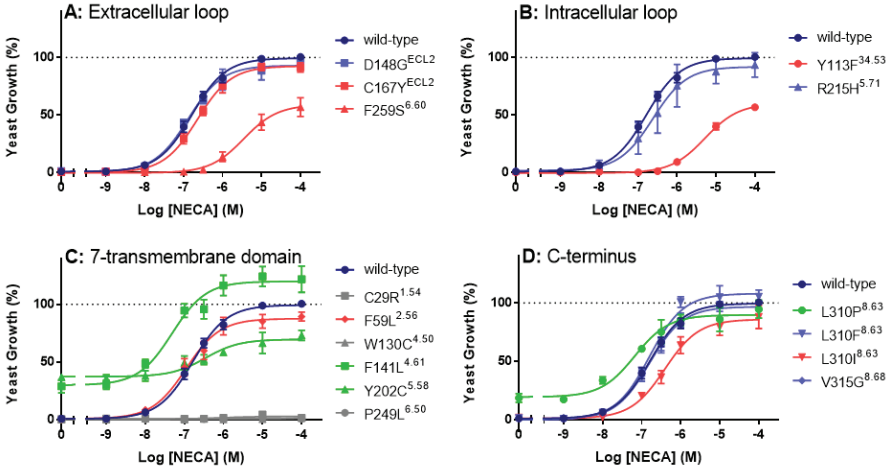

Figure 4. Concentration-growth curves for the wild-type and 15 cancer-related mutant A_{2B} receptors in response to the reference full agonist NECA. The maximum activation level of wild-type A_{2B} receptors was set at 100%, the background of the selection medium was set at 0%. The mutations are shown in the numbering of the A_{2B} receptors amino acid sequencing as well as according to the Ballesteros–Weinstein numbering[152]. The no-effect mutants are shown in blue, less active mutants in red, constitutively active mutants in green and loss of function mutants in grey. Reproduced with permission from Wang *et al*. (2020)[151].

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