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Lengger, Bettina; Jensen, Michael Krogh

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Engineering G protein-coupled receptor signalling in yeast for biotechnological and medical purposes

Bettina Lengger¹ and Michael K. Jensen^{1,*}

¹Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kgs. Lyngby, Denmark

* To whom correspondence should be addressed. Michael K. Jensen: Email: mije@biosustain.dtu.dk, Tel: +45 6128 4850

Abstract

G protein-coupled receptors (GPCRs) comprise the largest class of membrane proteins in the human genome, with a common denominator of 7-transmembrane domains largely conserved among eukaryotes. Yeast is naturally armoured with three different GPCRs for pheromone and sugar sensing, with the pheromone pathway being extensively hijacked for characterizing heterologous GPCR signalling in a model eukaryote. This review focuses on functional GPCR studies performed in yeast, and the elucidated hotspots for engineering and discusses both endogenous and heterologous GPCR signalling. Key emphasis will be devoted to studies describing important engineering parameters to consider for successful expression of functional coupling of GPCRs to the yeast mating pathway. We also review the various means of applying yeast for studying GPCRs, including the use of yeast armed with heterologous GPCRs as a platform for i) deorphanisation of orphan receptors, ii) metabolic engineering of yeast for production of bioactive products, and iii) medical applications related to pathogen detection and drug discovery. Finally, this review summarizes the current challenges related to expression of functional membrane bound GPCRs in yeast and discuss how opportunities to continue capitalising on yeast as a model chassis for functional GPCR signalling studies.

Keywords

GPCR, Yeast, Mating pathway, Biosensor

1. Introduction

Living cells respond to changes in environmental conditions and endogenous molecular events by means of interconnected signalling pathways, including regulatory proteins, metabolic enzymes, and receptors (Holsbeeks et al. 2004; Gupta et al. 2017). For cells to turn an input signal, whether extracellular or intracellular, into an adequate cellular output, cells rely on tight orchestration of the molecular components responsible for perceiving the input. In eukaryotes, one of the primary means for intra- and intercellular signalling is constituted by the G protein-coupled receptors (GPCRs)(Lagerström and Schiöth 2008; N. A. Brown et al. 2018). Found mainly in eukaryotes, GPCRs have evolved to recognize the diverse set of signalling cues contained within the intra- and extracellular environments of their hosts (Marinissen and Gutkind 2001). With 831 GPCRs encoded in the human genome, and being the target of roughly a third of all US Food and Drug Administration (FDA) approved drugs, GPCRs remain a focal point for both basic research and applied medical and biotechnological research communities (UniProt Consortium 2019; Hauser et al. 2017). Structurally, GPCRs are organized with 7 membrane-spanning alpha-helices, linked via 3 loops each extracellularly and intracellularly, with the N- and C-terminus exposed to the extracellular milieu and cytoplasm, respectively. Despite their common 7-transmembrane structure, GPCRs have evolved to sense a magnitude of different signals, including nutrients, pheromones, hormones, neurotransmitters, and light (Wacker, Stevens, and Roth 2017).

Yeast natively expresses three different GPCRs used for sugar and pheromone sensing (Versele, Lemaire, and Thevelein 2001). For *Saccharomyces cerevisiae*, glucose sensing is mediated by Gpr1 (Colombo et al. 1998; Kraakman et al. 1999), and pheromone-sensing by GPCRs Ste2 and Ste3 (Nakayama, Miyajima, and Arai 1985). Due to the ease of synthesizing peptide-based pheromones, one of the most extensively studied GPCR signaling cascades is the yeast pheromone pathway (R. Liu, Wong, and IJzerman 2016; Dohlman et al. 1991). Briefly, upon activation by one of the two *S. cerevisiae* produced a or a factor pheromones, yeast mating receptors Ste2 and Ste3 transduce the pheromone signal to the cell interior via the trimeric G protein consisting of the alpha subunit, Gpa1, the beta subunit, Ste4, and the gamma subunit, Ste18. Activation of the receptor induces the exchange of Gpa1-bound GDP to GTP, which in turn leads to separation of the $\beta\gamma$ -dimer from the α -subunit. Mating-specific responses are induced via $\beta\gamma$ -dimer coupled activation of the MAPK signalling cascade (Fig. 1A)(Leberer et al. 1992). Next, through Fus3-mediated phosphorylation, activation of the MAPK cascade causes the translocation of the Ste12 transcription factor to control the expression of more than 100

endogenous mating pathway target promoters (Fig. 1A)(Hung et al. 1997; Bardwell 2005; Roberts et al. 2000).

Due to the yeast mating pathway's resemblance to mammalian cell signaling (Pausch 1997; Versele, Lemaire, and Thevelein 2001), heterologous GPCRs from higher eukaryotes have often been expressed in yeast, and have furthermore been coupled to a reporter gene output to more broadly study GPCR signaling and use GPCRs for medical and biotechnological applications (Dohlman et al. 1991). Indeed, for more than three decades, yeast has been used as a platform to study the structure and function of endogenous and heterologous GPCRs (Nakayama, Miyajima, and Arai 1985; Byrne 2015; Ladds, Goddard, and Davey 2005), as a platform to find novel GPCR ligands and study cell-cell communication (Yasi et al. 2019; Billerbeck et al. 2018), for metabolic engineering purposes (Mukherjee, Bhattacharyya, and Peralta-Yahya 2015; Ehrenworth, Claiborne, and Peralta-Yahya 2017), and as a chassis for tuning and minimizing the complexity of GPCR signaling (Shaw et al. 2019). In general, yeast offers the opportunity of a synthetic "null" GPCR background for the study of non-native receptors due to ease of deleting *Ste2*, *Ste3*, and *Gpr1* (Dohlman et al. 1991; Andrew J. Brown et al. 2000; Pausch 1997). Moreover, for practical reasons, the shortened assay time compared to mammalian systems due to yeast's shorter doubling time and the non-requirement for cell passage is generally regarded as a benefit when studying GPCRs (Scott, Wybenga-Groot, et al. 2019). As such, in *Saccharomyces cerevisiae*, coupling of heterologous GPCRs to the *S. cerevisiae* mating pathway in order to express and functionally characterize GPCR signaling has been extensively reported (Dohlman et al. 1991; A. J. Brown et al. 2000; Pausch 1997). For GPCR structural studies on the other hand, *Pichia pastoris* has been a preferred chassis due to its high expression capacity of GPCR receptors needed for crystallisation studies (Byrne 2015), while *Schizosaccharomyces pombe* has also been reported in a few GPCR studies focused on GPCR signalling engineering and biosensor applications (Sasuga and Osada 2012; Ladds et al. 2003).

Here, we review strategies to facilitate heterologous GPCR expression and coupling to the native yeast mating pathway in *Saccharomyces cerevisiae* as well as expression strategies in *Pichia pastoris*. Special attention is given to functional GPCR signalling studies using reporter systems as proxies for GPCR signalling, as well as to the applications arising from the successful onboarding of synthetic GPCR signaling in yeast.

2. Design and engineering principles of GPCR signaling in yeast

The yeast mating pathway, naturally equipped to transduce signals from a GPCR to the cell interior, offers an engineering playground to achieve coupling of heterologous GPCRs to a reporter output (Shaw et al. 2019). Below, we provide a comprehensive review of how the natural yeast pathway has been adapted for studies related to receptor deorphanisation and signaling, as well as biotechnology or medical applications (Fig. 1A-B, sections 2.1-2.9).

The first functional heterologously expressed GPCR in yeast was based on the human $\beta 2$ adrenoceptor activated by its agonist isoproterenol (King et al. 1990). Since then, many more receptors have been coupled to the yeast mating pathway (R. Liu, Wong, and IJzerman 2016). GPCRs are commonly divided into 6 classes (class A-F), based on sequence homology, with only classes A-C, and F containing mammalian receptors ([Alexander et al. 2017](#)). Class A, the rhodopsin-like receptors, contains more than 80% of all GPCRs, and for the sheer number of receptors, this class is at the centre of research interest (Hu, Mai, and Chen 2017). Further division into subfamilies is based on receptor function and the receptor's specific ligand(s), or exclusively based on the nature of its ligands ([Davies et al. 2007](#); [Pándy-Szekeres et al. 2018](#)). This review follows the subclassification strategy used in the GPCRdb ([Pándy-Szekeres et al. 2018](#)), with a focus on the sequence-diverse human GPCRs which have been successfully functionally coupled, (Fig. 2). Indeed, human receptors of all subclasses in Class A, except steroid GPCRs, have been successfully expressed, including aminergic, peptide, protein, lipid, melatonin, nucleotide, alicarboxylic acid, sensory and orphan receptors (Fig. 2A, Suppl. Table S1)(Erlenbach, Kostenis, Schmidt, Hamdan, et al. 2001; Andrew J. Brown et al. 2000; King et al. 1990; Baranski et al. 1999; Bass et al. 1996; Zhang et al. 2002; Arias et al. 2003; Mukherjee, Bhattacharyya, and Peralta-Yahya 2015; Andrew J. Brown et al. 2011; Kokkola et al. 1998; Campbell et al. 1999; Chambers et al. 2000; R. Liu, van Veldhoven, and IJzerman 2016; Scott, Chen, et al. 2019; Huang et al. 2015). Additionally, a few GPCRs of the Class B1 has been functionally expressed (Fig. 2A)(Miret et al. 2002; Ladds et al. 2003; Kajkowski et al. 1997).

While engineering of all these receptors has the coupling of a heterologous GPCRs to the endogenous yeast mating pathway in common, the vast number of engineering attempts have highlighted important molecular and conditional parameters to consider for successful onboarding and studying. This section seeks to review the engineering hot-spots for rationalizing and streamlining GPCR signaling carried out over the last few decades. The content of sections 2.1-2.8 are structured according to the schematic outline shown in Fig 1B.

2.1. Engineered G protein-coupling

The key player in the coupling of heterologous GPCRs to the yeast mating pathway is the trimeric guanine nucleotide-binding protein (G protein), consisting of the three subunits $G\alpha$, $G\beta$ and $G\gamma$ (Syrovatkina et al. 2016). Exchange of GPCR-bound guanosine diphosphate (GDP) to guanosine triphosphate (GTP) activates the G protein, promoting the dissociation of the G protein from its receptor, and the separation of the $G\alpha$ subunit from the $G\beta\gamma$ dimer (Fig. 1)(Leberer et al. 1992). The dissociated subunits can now relay the signal further on to induce intracellular signalling events and an adequate response to the signal perceived (Marinissen and Gutkind 2001). As connecting entity, the G protein plays a crucial role as mediator between the heterologous receptor and the rest of the yeast mating pathway.

Generally, heterologous GPCRs show coupling preferences according to their native $G\alpha$ coupling. In humans, the genome encodes 16 $G\alpha$ subunits, which fall into 4 major classes, namely $G_{\alpha i/o}$, $G_{\alpha s}$ and $G_{\alpha q}$, $G_{12/13}$ (Syrovatkina et al. 2016; UniProt Consortium 2019). While coupling of heterologous GPCRs has been achieved using the endogenous yeast $Gpa1$ $G\alpha$ subunit (Mukherjee, Bhattacharyya, and Peralta-Yahya 2015), or the $G\alpha$ heterologous counterpart, chimeric $G\alpha$ subunits often couple more efficiently and are commonly used (Andrew J. Brown et al. 2000; Erlenbach, Kostenis, Schmidt, Hamdan, et al. 2001). Chimeric $G\alpha$ subunits typically consist of the native $G\alpha$ protein with C-terminal replacement of 5 amino acids, based on the studies describing that these amino acids play a crucial role in receptor recognition (Conklin et al. 1993; J. Liu et al. 1995). A large study investigated the coupling of eight receptors with seven chimeric $G\alpha$ subunits from three different $G\alpha$ subtypes. For seven out of eight GPCRs tested, coupling efficiency and ligand sensitivity improved when using chimeras (Andrew J. Brown et al. 2000). Interestingly, there seems to be a difference between different classes in terms of coupling promiscuity. More promiscuity is observed for receptors natively coupling to $G_{\alpha s}$ or $G_{\alpha q}$, while $G_{\alpha i}$ coupling receptors tend to be more selective in choice of their G protein (Okashah et al. 2019).

Beyond identification of matching GPCR: $G\alpha$ protein couples, optimizing the expression of G protein-encoding genes is an important parameter for successful GPCR signaling engineering. Indeed, optimally balanced levels for $G\alpha$, $G\beta$ and $G\gamma$, predicted using computational models (Bridge et al. 2018; Bush et al. 2016) and confirmed experimentally, ensure high pathway output while maintaining low basal activity of the pathway (Shaw et al. 2019).

Lastly, beyond balancing the expression of G protein subunits, the choice of $G\alpha$ has also been shown to be able to drastically affect agonist-sensing ability of olfactory receptors when expressed in yeast cells (Minic et al. 2005; Fukutani et al. 2012).

2.2. Streamlining GPCR signaling

Activation of the yeast pheromone pathway leads to initiation of mating (Versele, Lemaire, and Thevelein 2001). As mating genes are not required for studying GPCR signaling, and may even negatively affect functional outputs, elimination of certain GPCR signaling pathway-related genes is required to boost GPCR signalling strength, as exemplified by the negative regulator of Gpa1, SST2, and deletion of the *FAR1* gene, an inducer of cell cycle arrest during mating (Dohlman et al. 1996; Leplatois et al. 2001; Erlenbach, Kostenis, Schmidt, Serradeil-Le Gal, et al. 2001; Mukherjee, Bhattacharyya, and Peralta-Yahya 2015; Scott, Chen, et al. 2019).

More recently, Billerbeck *et al.* (2018), extended the minimizing of the pheromone response by also deleting *BAR1* encoding a protease cleaving α -factor into two inactive fragments exclude further negative feedback mechanisms, as well as the pheromone genes *MFA1/2* and *MFALPHA1/MFALPHA2* (Billerbeck et al. 2018). Streamlining efforts peaked with the engineering of an even further minimized and tuneable yeast strain, constructed by the knockout of 15 GPCR signalling genes, including all three native GPCRs (*STE2*, *STE3*, *GPR1*), negative regulators, inducers of cell cycle arrest (*FAR1*), and other genes involved in pheromone-based signaling (*MFA1/2* and *MFALPHA1/MFALPHA2*) (Fig. 1B). Moreover, by the use of defined 24 bp sequences for targeted insertion at the deletion sites, re-introduction of key signalling genes further allowed for cost-effective further engineering of a highly tunable GPCR signalling pathway (Shaw et al. 2019).

2.3. GPCR expression optimisation

Although successfully demonstrated with a number of GPCRs in yeast, expression of functional GPCRs is still far from trivial. Generally, receptor number is positively correlated with enhanced sensitivity (Bush et al. 2016; Shaw et al. 2019). High GPCR transcription can be obtained using strong promoters, as exemplified by the strong constitutive promoters like *TDH3* or *PGK1* (Billerbeck et al. 2018; Hashi et al. 2018). Likewise, the addition of the Kozak-like sequence -AAAAAAAUGUCU- upstream of a neurotensin GPCR open reading frame resulted in a 70% increased fluorescence reporter intensity upon stimulation with neurotensin agonist as compared to an isogenic strain without the Kozak-like sequence (Hashi et al. 2018; Hamilton, Watanabe, and de Boer 1987). However, despite these examples of transcriptional and post-transcriptional control, development of functional GPCR assays is often impeded by

unsuccessful processing or trapping of the GPCR in the endoplasmic reticulum (ER), cell surface non-availability, lack of accessory proteins, or presence of proteolytic spots in the receptor (O'Malley et al. 2009; Weston et al. 2015; Erlenbach, Kostenis, Schmidt, Serradeil-Le Gal, et al. 2001). Often, however, the reason for poor expression of GPCR is left undefined, and as such the reason for failure of processing and cell surface localization is unclear.

Especially affected by expression problems are olfactory receptors (ORs) (Lu, Echeverri, and Moyer 2003). This largely impacts OR deorphanisation studies, which is one of the reasons why currently only approx. 10% of the more than 400 human ORs have been deorphanized (Peterlin, Firestein, and Rogers 2014; UniProt Consortium 2019). In yeast, ORs which have been successfully expressed including human olfactory receptor 3A1 (OR17-40), seven ORs ectopically expressed in the colon, namely OR10S1, OR2A7, OR2L13, OR2T4, OR51B5, OR2A42, and OR2W3, the rat I7 OR, and the nematode ODR-10 (Fig. 2) (Minic et al. 2005; Yasi et al. 2019; Pajot-Augy et al. 2003; Tehseen et al. 2014). To circumvent poor expression, the well-expressing rat I7 receptor has served as an expression scaffold for ligand binding pockets of other GPCRs, including ORL829, ORL451, MOR226-1 (Radhika et al. 2007; Fukutani et al. 2012).

2.4. Leader sequences for membrane insertion and ER processing

To debottleneck the post-translational processing of GPCRs, N-terminal fusion of a leader sequence has been shown to aid insertion of the receptor into the plasma membrane (Fig. 1B) (Uddin et al. 2016). In the case of the yeast mating receptor Ste2, the 30 N-terminal residues ensure optimal signaling, are crucial for receptor cell surface expression, and responsible for negative regulation of GPCR signalling (Uddin et al. 2016). Early on, the functionalization of the β 2-adrenergic receptor was aided by the exchange of the receptor's N-terminal region with the corresponding sequence of the yeast endogenous Ste2 receptor (King et al. 1990). Studies in *P. pastoris* have also shown that the production level of the mouse 5-HT_{5A} serotonin receptor could be increased 3-fold when fusing the α -factor pre-propeptide to the receptor sequence (Weiss et al. 1998).

More recently, decreased fluorescence was observed for neurotensin receptor type 1 when adding a N-terminal sequence of the yeast mating receptor Ste2, while fluorescence remained at the level of cells carrying constructs without signal sequences when adding an N-terminal sequence of the invertase Suc2 (Bielefeld and Hollenberg 1992; Hashi et al. 2018). Furthermore, addition of pre-pro- and pre-sequences of α -factor protein as well as the N-terminal sequence of Ste2 to the somatostatin GPCR SSTR5 were investigated. Here, the addition

of any of these sequences was observed to improve expression. However, this did not translate to higher cell surface display, but did increase sensitivity of the receptor, although efficacy, defined as response created for a given amount of ligand, was decreased for all receptors studied (Iguchi et al. 2010).

In another GPCR fusion study, expression of 12 different GPCRs with attachment of a hydrophobic pre-prosequence was tested for better membrane insertion (O'Malley et al. 2009; Ng, Brown, and Walter 1996). For most receptors, higher expression levels could be achieved with the pre-prosequence, however for 11 of the 12 receptors, the primary location was still observed intracellularly. Upon investigation of the desired cleavage of the pre-prosequence in ER and Golgi, the majority had intact signal sequences, hinting to problems with protein processing, as further hypothesized by the activation of the unfolded protein response (O'Malley et al. 2009).

Fukutani *et al.* went on the same path for ORs by expressing mouse OR266/rat I7 chimeras. Here, the 32 and 58 N-terminal residues of mouse OR266 were replaced with the corresponding rat I7 sequence in an attempt to improve localization and signal transduction (Fukutani et al. 2012). Additionally, the 27 C-terminal residues of mouse OR226 residues were replaced with the corresponding 33 C-terminal residues of rat I7. From these designs, exchange alone lead to increased signal output, but bioluminescence counts could be almost doubled as compared to the native receptor by replacing both the C-terminal 33 and N-terminal 58 aa, which included an intracellular loop of the receptor (Fukutani et al. 2012).

Likewise, beyond N-terminal fusions and chimera strategies, intracellular portions of two human Frizzled-type receptors, Fz1 and Fz2, have been successfully expressed in a Ste2 backbone, with both synthetic GPCRs retaining functional G protein coupling and activation by mating factor in yeast (Dirnberger and Seuwen 2007). Moreover, internal loops of GPCRs have been speculated to be a target of proteases in yeast, thus leading to receptor degradation. Indeed, by deleting the central portion of the third intracellular loop of the M1, M3 and M5 muscarinic receptors, increased receptor amounts and increases in Bmax values have been observed (Erlenbach, Kostenis, Schmidt, Hamdan, et al. 2001). In relation to this, protease-deficient strains have also been used to express the dopamine D2 receptor and various other membrane proteins (Sander et al. 1994; Routledge et al. 2016).

2.5. Accessory proteins

Co-expression of odorant receptors with a receptor transporting protein have been demonstrated to aid transport of both the eugenol-responsive OREG from mice and the human androstenedione-responsive OR7D4 to the yeast cell membrane, while co-expression with odorant binding proteins from the silkworm moth *Bombyx mori* also increased sensitivity of OREG to eugenol (Fukutani et al. 2015). Other accessory proteins include the receptor-activity modifying proteins (RAMPs), which can associate with peptide hormone receptors of the Class B GPCRs and thereby modulate their activity (K. R. Klein, Matson, and Caron 2016). Moreover, it has been shown that interaction of receptors with RAMPs can alter their ligand specificity, transport to the cell membrane, internalisation, and even downstream signalling (K. R. Klein, Matson, and Caron 2016). Also noteworthy, while not reported in yeast, by expression of the RAMP1-dependent calcitonin receptor-like receptor CL1 with RAMP2 or RAMP3, CL1 has been reported to behave as an adrenomedullin receptor (Poyner et al. 2002), just as interaction of RAMPs with GPCRs can guide the receptor to the cell surface (Sexton et al. 2006).

While the mechanism for the mode-of-actions of many accessory proteins can be far from generalized, for a more in-depth overview of other accessory proteins, including guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs), GTPase-activating proteins (GAPs), and G β γ -interacting proteins, the reader is referred to Sato *et al.* (Sato et al. 2006; Sato 2013).

2.6. Synthetic transcription factors and promoters

Activation of the MAPK cascade causes the translocation of the Ste12 transcription factor from the cytoplasm to the nucleus to regulate the expression of more than 100 endogenous mating pathway target promoters (Roberts et al. 2000). Indeed, the use of Ste12 controlling reporter gene expression from either of the *FUS1*, *FUS2* and *FIG1* promoters remains the most common design for analysing GPCR signaling in yeast (Muller et al. 2003; Trueheart, Boeke, and Fink 1987; Trueheart and Fink 1989; Alvaro and Thorner 2016), though it is important to limit the expression strength of Ste12 in order not to impair cell growth (Fig. 1B)(Shaw et al. 2019).

Alternatively, uncoupling heterologous GPCR signalling from the native mating pathway output can be obtained by the use of synthetic transcription factors, thereby enabling targeted transcription of the reporter gene without affecting endogenous mating pathway target promoters. In a recent study, the pheromone-responsive domain (PRD) of native Ste12

transcription factor was combined with the full-sequence *LexA* bacterial repressor protein (Shaw et al. 2019; Mukherjee, Bhattacharyya, and Peralta-Yahya 2015). The reporter gene was under the control of a synthetic promoter with a variable number of *lexA* DNA binding domains, enabling the modulation of the output response over a 3-fold range corresponding to the number of *lexA* DNA binding domains, while maintaining a tight low basal expression state (Shaw et al. 2019). Similarly, for the coupling of the olfactory OR1G1 GPCR coupling to yeast, the use of the *PLexA(4x)* promoter for GFP expression showed a 7-fold increase in the presence of decanoic acid as compared to GFP expression under the *FIG1* promoter controlled by Ste12 (Mukherjee, Bhattacharyya, and Peralta-Yahya 2015).

2.7. GPCR signaling read-outs

Choice of reporter assay for functional GPCR screens is most often associated with growth, fluorescence, colourimetric or phenotypic screens like beta-galactosidase and carotenoid (Price et al. 1995; Shaw et al. 2019; Ostrov et al. 2017).

A HIS3 growth coupled assay has been used for screening for weak partial agonists and inverse agonists (i.e. GPCR ligands shifting the equilibrium toward the inactive state) of constitutively active GPCRs, like chemokine receptor mutant CXCR4(N119S) responsive to stromal-derived-factor-1 (Zhang et al. 2002; Evans et al. 2009). Complementary to this, inverted reporter systems have been used for detection of non-functional GPCR mutants and detection of antagonists as in the case of rat M3 muscarinic acetylcholine receptor (M3R) expressed in yeast (B. Li et al. 2007). Here, the FUS2-controlled expression of CAN1 was triggered by carbachol-induced activation of M3R. With CAN1 encoding a permease allowing the import the cytotoxic canavanine, only non-functional mutated receptors would survive supporting the analysis of functionally critical amino acids (B. Li et al. 2007; Scarselli et al. 2007). Similar to CAN1, FUI is a permease for 5-fluoropyrimidine permease, and when using toxic 5-fluorouridine (5-FU), inhibition of FUI expression will result in survival of yeast cells expressing non-functional GPCRs stimulated with inverse agonists (Evans et al. 2009).

With respect to fluorescent reporter read-outs, an inverted fluorescence reporter for detection of hSSTR5 somatostatin agonists has been established in which activation of the yeast mating pathway by an agonist blocks the expression of fluorescent ymUkG1, while an antagonist present at the receptor will displace the agonist, leading to receptor inactivation and expression of ymUkG1 (Fukuda et al. 2011).

Overall, for functional assays of GPCR signaling in yeast, the trend goes towards the use of novel, yeast-optimized fluorescent markers with high sensitivity and high signal-to-noise ratios (Nakamura, Ishii, and Kondo 2013; Hashi et al. 2018; Kaishima et al. 2016).

2.8. Evolution of GPCRs

With advanced genetic tools and screening systems, we no longer solely rely on rational engineering of naturally occurring GPCRs. Rounds of mutagenesis coupled with selection allows for directed evolution of GPCRs with optimized expression levels, improved ligand sensitivity, and altered ligand specificities (Adeniran et al. 2018; Schütz et al. 2016).

In one of the earliest studies of using directed evolution for altering ligand specificity, mutations in *S. cerevisiae* mating receptor Ste2 were shown to allow recognition of *Saccharomyces kluyveri* pheromone ligands (Marsh 1992), whereas random and site-directed mutagenesis of the Ste2 receptor has also enabled receptor variants to specifically and strongly recognize the *Kluyveromyces lactis* pheromone (Di Roberto, Chang, and Peisajovich 2017). Supposedly, the change in specificity arises from enhanced binding affinity to the foreign pheromone, or due to decreased interactions with the negative regulator Sst2 (Fig. 1) (Di Roberto et al. 2016). In other examples yeast has served as a platform to evolve acetylcholine-responsive muscarinic acetylcholine receptors for potent and specific activation by the inert pharmaceutical compound clozapine-N-oxide (Armbruster et al. 2007), whereas Dong *et al.* describe evolution-guided engineering of GPCRs without affinity to their endogenous ligand(s), no basal activity, and potent and exclusive activation of the GPCR by another candidate inert pharmaceutical compound (Dong, Rogan, and Roth 2010). Obviously, evolution-guided engineering of new ligand specificities have not shied away from medical research interest. As such, Ste2 underwent a substrate walking approach involving chimeric intermediate ligands to specifically recognize the biomarker protein Cystatin C (Adeniran et al. 2018).

Other novel receptors showing activation by synthetic compounds are termed RASSLs (receptors activated solely by a synthetic ligand), DREADDS (designer receptors exclusively activated by designer drugs), therapeutic receptor-effector complexes (TRECes) or neoceptors (Conklin et al. 2008). Collectively these synthetic GPCRs are considered important platforms for inferring drug targetability in the development of new medicine.

Finally, directed evolution has been demonstrated useful for increasing GPCR expression, with best-performing variants having up to 26-fold improved expression levels (Schütz et al. 2016).

While this study was demonstrated in insect cells, the workflow should be easily adaptable to ameliorate receptor expression in yeast.

2.9. Other improvement strategies

Beyond the genetic design parameters for engineering GPCR signalling in yeast, it has been shown that lipid composition of yeast membranes can impact expression of a GPCR. To make yeast cell membranes more human-like, ergosterol synthesis has been replaced with cholesterol, and demonstrated the functional expression of Ste2 and other membrane proteins (Routledge et al. 2016; Hirz et al. 2013; Morioka et al. 2013). Also, in *P. pastoris* culture conditions like incubation time and temperature after induction were tested in order to identify optimal conditions for expression of taste receptors (Sugawara et al. 2009). Here, cultivation at 20 °C was found to improve GPCR expression, speculatively due to decreased protein synthesis rates and in turn, production of properly folded proteins.

3. GPCR studies in yeast for biotechnological and medical applications

While traditionally yeast has played a major role in expression of GPCRs for crystallization studies and mutagenesis studies for sequence-structure explorations (Byrne 2015), this section will discuss some of the more recent demonstrations focusing on yeast as a key chassis in the development of GPCR-based biosensors for metabolic engineering applications and point-of-care environmental monitoring, as well as a platform GPCR deorphanisation studies (Fig. 3).

3.1. Novel ligands and deorphanisation studies

With the first heterologous GPCR coupled to the yeast mating pathway, it became immediately apparent that this signalling pathway harboured great potential for the discovery of novel ligands (King et al., 1990, Price et al., 1995).

Following the first demonstration of heterologous expression of GPCRs in yeast, somatostatin analogs with a L-Cys₂-LCys₇ pair were identified as potent agonists, while similar D-Cys₂,L-Cys₇ had antagonistic properties, which was in accordance with a study in mammalian

cells (Fig. 3A)(Bass et al. 1996). For the human formyl peptide receptor like-1 (FPRL-1) receptor, six novel peptide agonists from a random plasmid library were identified, and screening of a pyrrolopyrimidines library against two adenosine receptors yielded two novel antagonists (Fig. 3A)(C. Klein et al. 1998; Campbell et al. 1999). Studies by Brown *et al.* showed that propionate and further short chain carboxylic acids have agonistic effects on at-that-time orphan receptors GPR41 and GPR43 (Andrew J. Brown et al. 2003).

Also, in a search for novel antagonists to be used in combination-therapy together with allosteric agonists, a library of 160,000 chemokine ligands was screened in search for novel allosteric CXCR4 antagonists (Sachpatzidis et al. 2003). The authors identified two peptide agonists named RSVM and ASLW as lead compounds for further pharmaceutical development and additionally showed the existence of alternative agonist-binding spots in the CXCR4 receptor (Sachpatzidis et al. 2003). Additionally, a point mutation at position N119 of the CXCR4 receptor rendered it constitutively active, and made it possible to identify T140 as an inverse agonist, and AMD3100 and ALX40-4C as weak partial agonists (Zhang et al. 2002).

For GPR119, a suggested target for obesity and diabetes type 2 treatment, novel agonist PSN375963 with potency similar to the suggested endogenous ligand oleoylethanolamide (OEA) was found (Overton, Fyfe, and Reynet 2008; A. J. Brown 2007). Also, the benzodiazepine lorazepam was found to be non-selective agonist of GPR68 in yeast-based assays (Fig. 3A)(Huang et al. 2015).

In one of the early deorphanisation studies of ORs in yeast, the orphan receptor KIAA0001, nowadays known as P2Y purinoceptor 14, was expressed together with different G protein α -subunits, ultimately identifying UDP-Glucose as a specific agonist P2Y purinoceptor 14 (Chambers et al. 2000). Still the role of most ORs remains to be established as only 10% of the approx. 400 ORs, have been deorphanized (Fig. 2A-B)(Flegel et al. 2013), yet expression constraints in heterologous systems, together with odorant insolubility, have hindered a thorough characterisation of these receptors (Mukherjee, Bhattacharyya, and Peralta-Yahya 2015). In the largest deorphanisation attempt in a yeast system, Yasi *et al.* expressed seven human olfactory receptors in yeast (Yasi et al. 2019). Due to their orphan status, functional coupling of the receptor to the pathway was not possible to validate, and immunofluorescence microscopy was therefore used to determine localisation as a proxy for functional expression. In a medium-throughput screen, yeast served as an adequate platform to screen seven ORs against 57 chemicals expected to be found in olfactory tissues. Screening resulted in the deorphanisation of two receptors, namely OR2T4 (α -pinene, lilial, and undecanal) and OR10S1 (lilial), as well as the identification of α -pinene and lilial as novel ligands for OR2A7,

emphasizing the value of yeast-based screening system also for olfactory receptor deorphanisation (Fig. 3A)(Yasi et al. 2019).

Finally, coming back to the native mating pathway in yeast, library screening of yeast mating factor variants found that Ste2-binding agonists and antagonists preferably include histidine, leucine, leucine, proline, a nonaromatic hydrophobic residue, and an aromatic residue at positions 2, 4, 6, 11, 12, and 13, respectively (Manfredi et al. 1996).

3.2. Biotechnological applications

In addition to yeast's proven record as a screening system for deorphanisation and novel ligand identification, yeast-based systems are also highly relevant as screening tools for improving microbial cell factories. Microbially produced colourimetric products, such as carotenoids or certain polyketides, can be easily detected using spectrophotometry, and as such be easily linked to cell factory optimization (Tong et al. 2015; Jakočiūnas et al. 2015). However, the majority of products synthesized in yeast which are of biotechnological interest are colourless, and therefore restricted to semi-throughput LC/- or GC/MS-based quantification methods. For this purpose major efforts in the metabolic engineering and synthetic biology communities focus on the development of generally applicable designs for higher-throughput screening technologies, including transcription factor-based biosensors for detection of small-molecule accumulations in whole-cell biocatalysts like yeast (Umeyama, Okada, and Ito 2013; Taylor et al. 2016; Skjoedt et al. 2016).

Mitigating the limits of transcription factor-based biosensors for small-molecule detection, Radhika *et al.* (2007) introduced the concept of developing GPCR-based biosensors in yeast for detection of chemical landscapes beyond the capacity of transcription factor-based biosensors (Radhika et al. 2007). Here the authors engineered yeast to detect 2,4-dinitrotoluene, an explosive mimic, using the rat olfactory receptor Olfr226 (Radhika et al. 2007). Contrary to the majority of GPCR-biosensors, the biosensor was the first one to be built using the complete cAMP system with heterologous key parts including a fluorescent reporter instead of the yeast mating pathway. Moreover, Mukherjee *et al.* (2015) developed GPCR-based biosensors for facile detection of medium chain-fatty acids based on GPR40, a fatty acid receptor, and OR1G1, an olfactory receptor for future use in yeast cell factory optimization (Fig. 3B)(Mukherjee, Bhattacharyya, and Peralta-Yahya 2015). Medium chain fatty acids are immediate precursors of fatty acid methyl esters which can be microbially produced as replacement products for D2 diesel, and are therefore of great biotechnological relevance (Fig.

3B)(Peralta-Yahya et al. 2012). The study furthermore exemplified the development of a biosensor strain with a 13-17 fold signal increase upon GPCR activation with medium chain fatty acids, while also introducing the sender-receiver concept, in which a sender cell is producing the desired product, and a receiver (i.e. sensor) cell is activating the GPCR-based biosensor as a proxy for the production and secretion of the ligand from the sender cell (Mukherjee, Bhattacharyya, and Peralta-Yahya 2015). The sender-receiver concept has also been successfully demonstrated in serotonin-producing yeast strains (Ehrenworth, Claiborne, and Peralta-Yahya 2017). Here, a human 5-HT₄ GPCR-based sensor strain showed a 2-fold increase in GFP signal in the presence of serotonin (Ehrenworth, Claiborne, and Peralta-Yahya 2017). Demonstration of the concept was done by medium-throughput screening of serotonin producing strains in a 96-well format by bringing sensing cells into contact with the spent media of the producing strains (Fig. 3B).

Likewise, whole-cell biosensors were developed to specifically measure melatonin production in melatonin producing strains expressing MTNR1A melatonin receptor (Shaw et al. 2019). Importantly, a consortium combining two sensing strains with different linear ranges of detection could cover the range of melatonin concentrations over four orders of magnitude produced in engineered melatonin-producing yeast strains (Fig. 3B)(Germann and Baallal Jacobsen 2016; Shaw et al. 2019). Most importantly, melatonin concentrations determined with the MTNR1A-2-strain biosensor consortium from the supernatant of 88 melatonin producing yeast strains were validated by LC-MS quantifications (Shaw et al. 2019).

3.3. Medical applications

Beyond metabolic engineering applications, GPCR-based biosensors have been developed for medical applications as well as environmental monitoring. Human pathogenic and agricultural or food spoiling yeast species produce diverse mating pheromones (Seike, Shimoda, and Niki 2019). Making use of this concept, biosensors for pheromones of *Candida albicans*, *Candida glabrata*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, *Lodderomyces elongisporus*, *Botrytis cinerea*, *Fusarium graminearum*, *Magnaporthe oryzae*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces rouxii* have been created based on their respective yeast mating receptors (Ostrov et al. 2017). Ligand detection was coupled to an easily readable colourimetric response with detection in the nanomolar range thereby enabling detection of pathogenic yeasts in complex environmental samples (Ostrov et al. 2017). For detection of the *P. brasiliensis* strain, sensitivity was further improved to allow detection with picomolar sensitivity by controlling the GPCR expression levels using the strong *CCW12* promoter.

Additionally, and as already mentioned in section 2.8., the Ste2 GPCR was evolved to detect Cystatin C, a biomarker for chronic kidney disease at 50 μM sensitivity in human urine (Adeniran et al. 2018). Interestingly, coupling of the light-sensing human rhodopsin to the yeast mating pathway was achieved more recently (Scott, Chen, et al. 2019). With respect to the latter, activation of the rhodopsin receptor by its endogenous activator light provides a prime model for the study of mutant rhodopsins, involved in causing eye diseases (e.g. retinitis pigmentosa). Indeed, the P23H mutant rhodopsin, which represents a common disease-causing mutation, has been expressed in yeast, and used to screen for potential rhodopsin-stabilizing compounds with the ultimate goal of developing a drug to combat vision loss and ameliorating disease (Scott, Wybenga-Groot, et al. 2019). Despite no suitable compounds have so far been identified, this study elegantly demonstrates cost-efficient ligand screening in a semi-high-throughput format (Fig. 3C)(Scott, Chen, et al. 2019; Scott, Wybenga-Groot, et al. 2019).

4. Outlook

As reviewed herein, more than 50 GPCRs have been functionally expressed in yeast (Fig. 2, Suppl. Table S1)(Shaw et al. 2019). As guiding principles, for functional coupling of heterologous GPCRs, N-terminal receptor fusions with signaling sequences have been shown to enhance the expression of several receptors, and G_{α} coupling specificity have also been reasonably well-established. Likewise, removal of a loop for muscarinic receptors ameliorates their expression, and for some GPCRs, helper proteins guide their translocation to the outer membrane. Moreover, the key molecular features for tuning response-curves of GPCR-based biosensors has been established, with critical focus towards increasing GPCR numbers for improved sensitivity, reduction of leaky basal activity by G protein expression optimisation, and adoption of synthetic transcription factors and engineered promoters to tune the dynamic output range (Shaw et al. 2019; Mukherjee, Bhattacharyya, and Peralta-Yahya 2015). However, challenges remain in terms of post-translational processing, expression issues, and at the level of G protein-coupling (Sarramegna et al. 2003; Routledge et al. 2016), underscoring that while the minimized and tunable molecular blueprint for yeast mating pathway is well-established, the onboarding of many heterologous GPCRs into yeast remains a demanding task with yet-to-be established more general design principles.

Still, the current state-of-the-art within design, engineering and applications of GPCR signaling, has underscored yeast as a valuable resource for functional GPCR studies (Adeniran et al. 2018; Ehrenworth, Claiborne, and Peralta-Yahya 2017; Ostrov et al. 2017; Mukherjee,

Bhattacharyya, and Peralta-Yahya 2015; Shaw et al. 2019), as well as a screening platform for metabolic engineering and medical purposes (Mukherjee, Bhattacharyya, and Peralta-Yahya 2015; Ehrenworth, Claiborne, and Peralta-Yahya 2017; Shaw et al. 2019; Adeniran et al. 2018; Scott, Wybenga-Groot, et al. 2019). Beyond this, the means to study GPCR signaling in a “null” yeast background, together with the low cost of cultivation and the amenable toolkit for genome engineering and directed evolution, underscores yeast as being well-suited for high-throughput deorphanisation and novel ligand studies using diverse compound libraries (Yasi et al. 2019; Scott, Wybenga-Groot, et al. 2019). Indeed, for GPCR deorphanisation studies, toxicity studies have been performed and have found bioactive ligands of GPCRs to be well tolerated in yeast at nM-mM concentration ranges used for yeast-based assays (Ostrov et al. 2017; Jarque, Bittner, and Hilscherová 2016), and pharmacokinetic studies (Andrew J. Brown et al. 2011). Ultimately, the enormous potential for drug discovery and GPCR engineering based on semi-throughput, low-cost, and sensitive assays in yeast (Scott, Wybenga-Groot, et al. 2019; Mukherjee, Bhattacharyya, and Peralta-Yahya 2015), together with new tools for studying GPCR deorphanisation using RNA-sequencing in combination with multiplexed barcoding strategies is expected to fast-forward identification of ligands for orphan GPCRs in high-throughput (Jones et al. 2019). Additionally, considering the ease by which peptide libraries can be secreted from yeast (Shigemori, Kuroda, and Ueda 2015), it is now feasible to perform large compound library screens within biologics which can then be assayed combinatorially against libraries of orphan GPCRs.

Furthermore, it should also be mentioned that yeast provides a powerful and versatile chassis for metabolic engineering applications for the production of bioactive natural products like cannabinoids and opiates (Nielsen and Keasling 2016; Siddiqui et al. 2012; Luo et al. 2019; Galanie et al. 2015). Metabolic engineering of bioactive natural products often includes balancing gene expression of biosynthetic pathways, expansive pathway optimisation based on homology searches, codon-optimisations, and compartmentalization strategies (Galanie et al. 2015; S. Brown et al. 2015; Luo et al. 2019). Taken together, this makes up a vast multi-parametric solution space, for which the capacity to design and construct refactored biosynthetic pathways far outstrips the screening capacity for said molecules. Exactly for this purpose, GPCRs have already been harnessed for facile and semi-throughput screens of small libraries of natural product producing yeast strains (Ehrenworth, Claiborne, and Peralta-Yahya 2017; Shaw et al. 2019), and as such it is anticipated that the ongoing efforts to minimize and potentiate GPCR-mediated reporter assays will enable new means for high-throughput screening of biosynthetic pathway libraries for bioactive compounds. Furthermore, as for the biosynthesis of new-to-nature drugs in plants (Moses et al. 2014; Runguphan, Qu, and O'Connor

2010), and derivatized substrates in yeast (McCoy and O'Connor 2006; Y. Li et al. 2018), development and application of GPCR-based biosensors is expected to further potentiate pathway discovery and evolution-guided optimisation of whole-cell biocatalysts of completely new compound libraries which may have novel biological activities and potentially improved, pharmacological properties (Runguphan, Qu, and O'Connor 2010). Beyond the potential environmental benefit with respect to biobased manufacturing of natural products, and the societal impacts derived from new and improved therapies toward human illnesses, these studies should also help establish more broadly applicable expression guidelines to further advance GPCR signaling studies in general.

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6. Conflict of interest

The authors declare that they have no conflict of interest.

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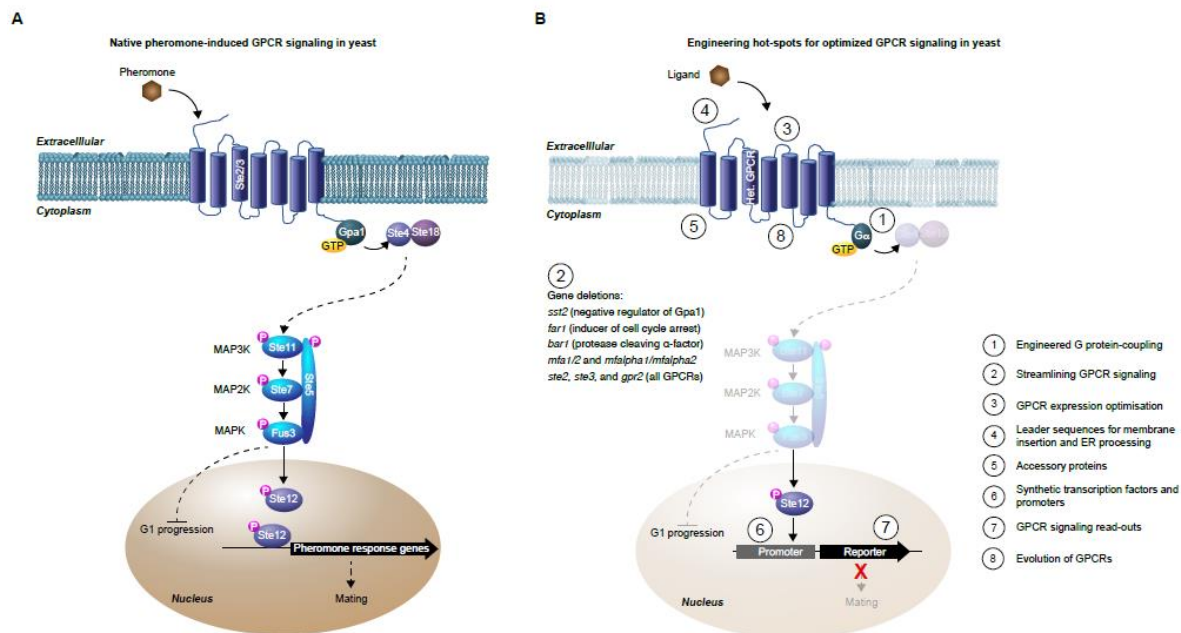


Figure 1.

Figure 1. The native pheromone-induced GPCR signalling pathways of wild-type and engineered yeast *Saccharomyces cerevisiae*. (A) The native pheromone-induced signalling pathway mediated by yeast GPCRs, Ste2 and Ste3, in wild-type yeast *Saccharomyces cerevisiae*. In the native pathway, the GPCR ligand, α - or a-factor, is bound by Ste2 or Ste3, respectively. This leads to exchange of GDP to GTP on the $G\alpha$ subunit, Gpa1, of the heterotrimeric G protein, and subsequently dissociation of the $G\beta$: $G\gamma$ dimer, Ste4 and Ste18, from the $G\alpha$ subunit. Release of Ste4:Ste18 dimer activates a MAP-kinase cascade, targeting the transcription factor Ste12 for phosphorylation, and subsequently translocation to the nucleus and induction of pheromone responsive genes required for yeast mating. (B) An engineered signaling pathway in yeast expressing a heterologous GPCR (Het. GPCR). In this pathway all major engineering hotspots of the signaling pathway are numbered and engineering strategy listed. Importantly, when expressing heterologous GPCRs in yeast, the native GPCRs Ste2, Ste3 and Gpr1 are usually deleted to avoid signaling crosstalk. Ultimately, activation of the engineered core pheromone pathway using coupled heterologous GPCRs activates reporter genes most often encoding gene products for growth on selective media or fluorescence read-outs.

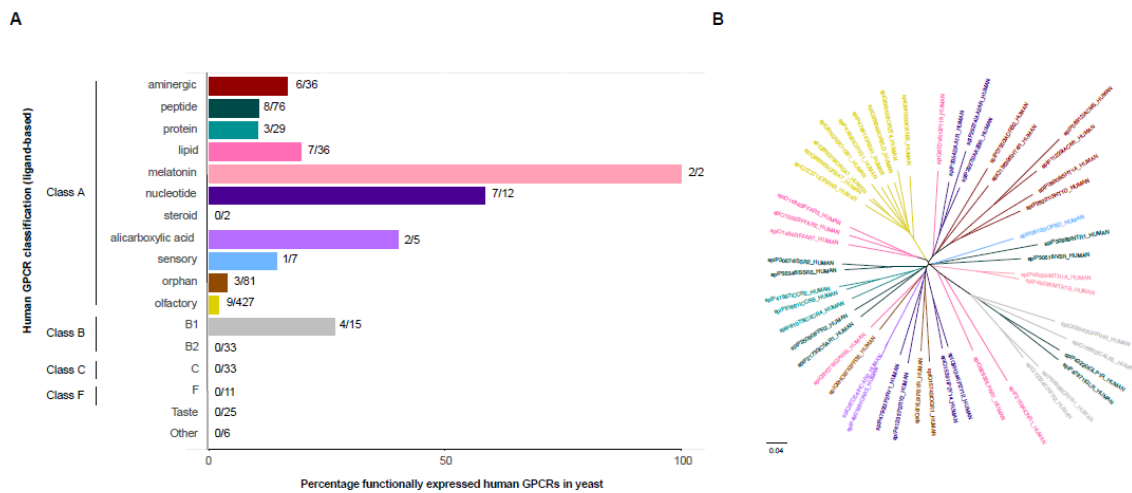


Figure 2. Human GPCRs functionally coupled to the yeast pheromone pathway. (A) A barplot visualizing the human GPCR classes and subtypes according to GPCRdb and UniProt (UniProt Consortium 2019; Pándy-Szekeres et al. 2018), and the percentage of those which have been published to be functionally coupled to the pheromone signaling pathway in yeast (see Suppl. Table. S1). Ratios indicate absolute numbers of GPCRs functionally expressed in yeast out of the total number of GPCRs found in that subclass. **(B)** Dendrogram displaying the phylogenetic relationship of human GPCRs functionally expressed in yeast. Colour-coding is aligned with GPCR colour-coding presented in (A).

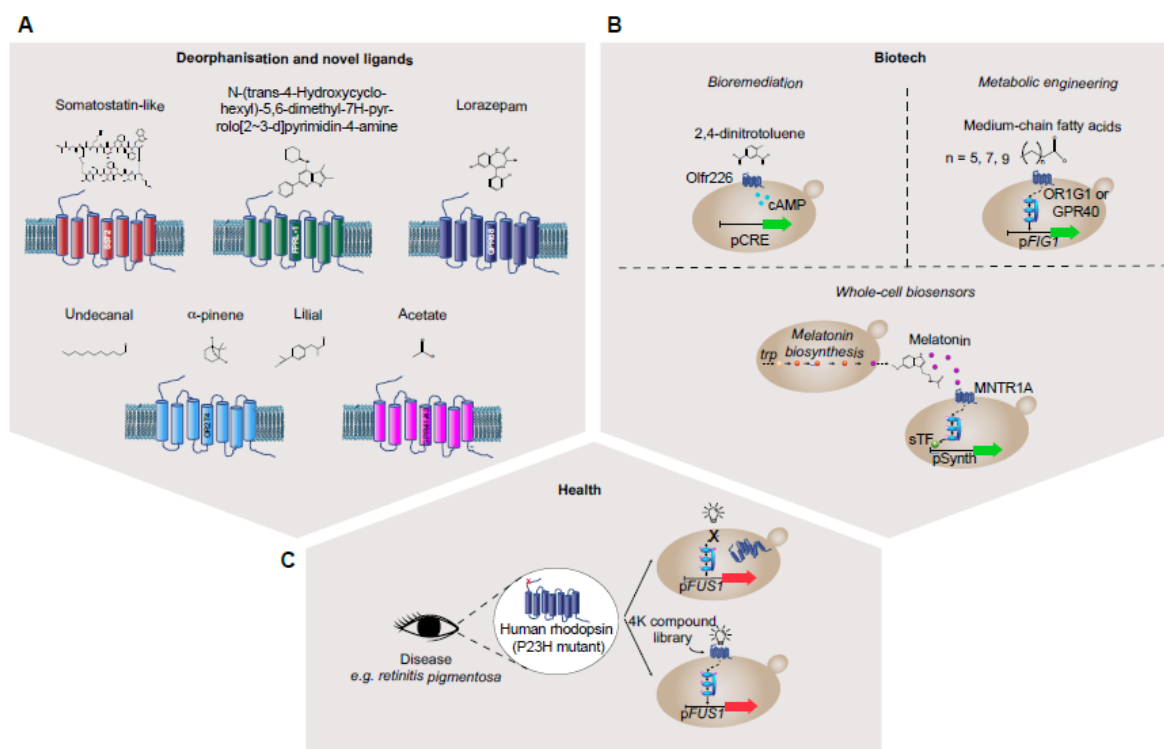


Figure 3. Applications using yeast for GPCR studies. (A) Examples in which yeast used as a key enabling technology for GPCR deorphanisation studies and for identification of novel ligands. (B) Within biotechnology functional expression of GPCRs in yeast has enabled the development of GPCR-based biosensors for point-of-care environmental monitoring and semi-throughput screens for metabolic engineering applications. (C) For health applications and medical research yeast has been adopted for compound screens to search for stabilising disease-causing GPCR variants. pCRE = promoter with a cAMP responsive element (CRE). pFUS1 = native promoter driving the expression of *FUS1*. pFIG1 = native promoter driving the expression of *FIG1*. pSynth. = synthetic promoter. sTF = synthetic transcription factor. Green and red arrows indicate expression cassettes for genes encoding GFP and mCherry, respectively.