MINIREVIEW

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

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One sentence summary: G protein-coupled receptors constitute one of the largest classes of sensory components in eukaryotes, and yeast offers a robust and simple platform for both GPCR signalling characterisation and biotechnological and medical applications. PRE = pheromone responsive element

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ABSTRACT

G protein-coupled receptors (GPCRs) comprise the largest class of membrane proteins in the human genome, with a common denominator of seven-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 characterising heterologous GPCR signalling in a model eukaryote. This review focusses on functional GPCR studies performed in yeast and on 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 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 summarises the current challenges related to expression of functional membrane-bound GPCRs in yeast and discusses the opportunities to continue capitalising on yeast as a model chassis for functional GPCR signalling studies.

Keywords: GPCR; yeast; mating pathway; biosensor

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; Brown *et al.* 2018). Found mainly in eukaryotes, GPCRs have evolved to recognise the diverse set of signalling cues contained within the environment 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 the US Food and Drug Administration (FDA) approved drugs, GPCRs remain a focal point for both basic research and applied medical and biotechnological research communities (Hauser *et al.* 2017; UniProt Consortium 2019). Structurally, GPCRs are organised with seven membrane-spanning alpha-helices, linked via three loops each
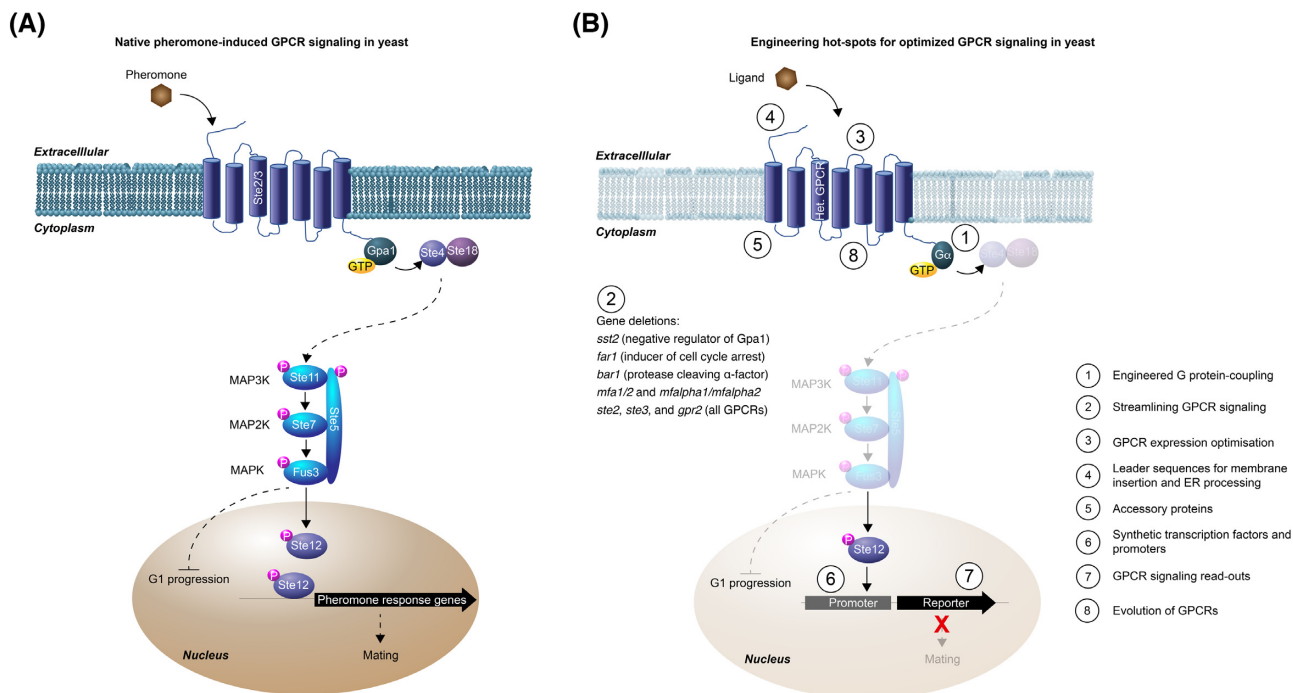
Figure 1. The native pheromone-induced GPCR signalling pathways of wild-type and engineered yeast *S. cerevisiae*. **(A)** The native pheromone-induced signalling pathway mediated by yeast GPCRs, Ste2 and Ste3, in wild-type yeast *S. cerevisiae*. In the native pathway, the GPCR ligand, α- or a-factor, is bound by Ste2 or Ste3, respectively. This leads to exchange of GDP to GTP on the Gα subunit, Gpa1, of the heterotrimeric G protein, and subsequently dissociation of the Gβ:Gγ dimer, Ste4 and Ste18, from the Gα subunit. Release of Ste4:Ste18 dimer activates an 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 signalling pathway in yeast expressing a heterologous GPCR (Het. GPCR). In this pathway, all major engineering hotspots of the signalling 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 signalling 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.

extracellularly and intracellularly, with the N- and C-terminus exposed to the extracellular milieu and cytoplasm, respectively. Despite their common seven-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 synthesising peptide-based pheromones, one of the most extensively studied GPCR signalling cascades is the yeast pheromone pathway (Dohlman *et al*. 1991; Liu, Wong and IJzerman 2016). Briefly, upon activation by one of the two *S. cerevisiae* produced a- or α-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 guanosine diphosphate (GDP) to guanosine triphosphate (GTP), which in turn leads to separation of the βγ-dimer from the α-subunit. Mating-specific responses are induced via βγ-dimer coupled activation of the mitogen-activated protein kinase (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 >100 endogenous mating

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

Due to the yeast mating pathway's resemblance to mammalian cell signalling (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 signalling 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; Ladds, Goddard and Davey 2005; Byrne 2015), as a platform to find novel GPCR ligands and study cell–cell communication (Billerbeck *et al*. 2018; Yasi *et al*. 2019), for metabolic engineering purposes (Mukherjee, Bhattacharyya and Peralta-Yahya 2015; Ehrenworth, Claiborne and Peralta-Yahya 2017), and as a chassis for tuning and minimising the complexity of GPCR signalling (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; Pausch 1997; Brown *et al*. 2000). 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 *et al*. 2019b). As such, in *S. cerevisiae*, coupling of heterologous GPCRs to the *S. cerevisiae* mating pathway in order to express and functionally characterise GPCR signalling has been extensively
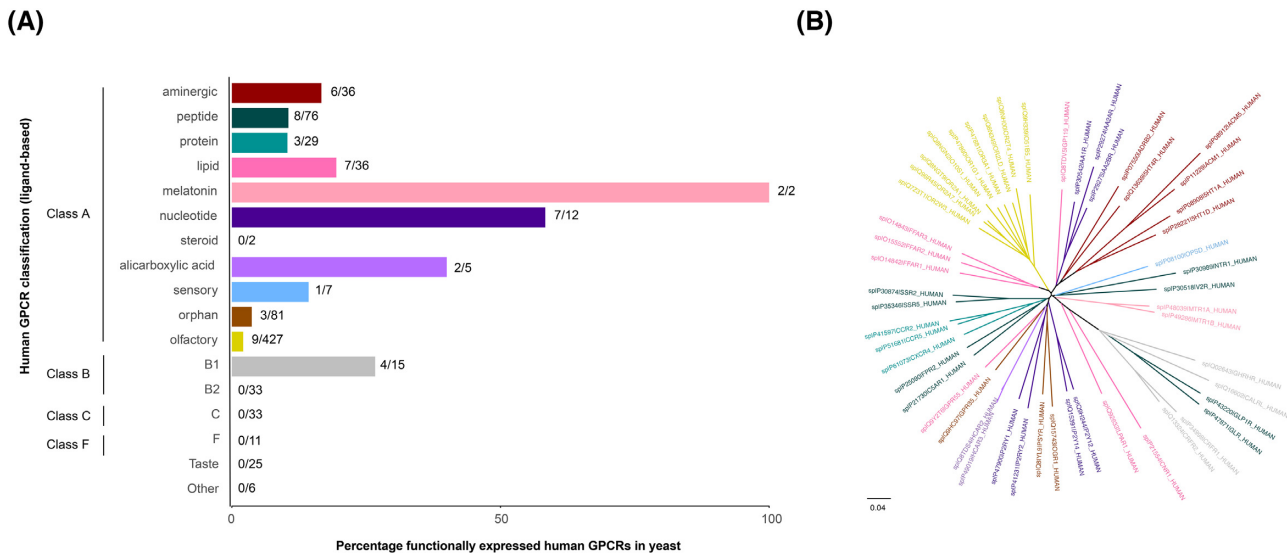
(A)

(B)

Figure 2. Human GPCRs functionally coupled to the yeast pheromone pathway. **(A)** A bar plot visualising the human GPCR classes and subtypes according to GPCRdb and UniProt (Pándy-Szekeres *et al.* 2018; UniProt Consortium 2019), and the percentage of those that have been published to be functionally coupled to the pheromone signalling pathway in yeast (see Table S1, Supporting Information). 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).

reported (Dohlman *et al.* 1991; Pausch 1997; Brown *et al.* 2000). 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 focussed on GPCR signalling engineering and biosensor applications (Ladds *et al.* 2003; Sasuga and Osada 2012).

Here, we review strategies to facilitate heterologous GPCR expression and coupling to the native yeast mating pathway in *S. cerevisiae* as well as expression strategies in *P. 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 signalling in yeast.

DESIGN AND ENGINEERING PRINCIPLES OF GPCR SIGNALLING 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 signalling, as well as biotechnological or medical applications (Fig. 1A and B; sections from 'Engineered G protein-coupling' to 'Other improvement strategies').

The first functional heterologously expressed GPCR in yeast was based on the human $\beta2$ adrenoceptor activated by its agonist isoproterenol (King *et al.* 1990). Since then, many more receptors have been coupled to the yeast mating pathway (Liu, Wong and IJzerman 2016). GPCRs are commonly divided into six classes (classes A–F), based on sequence homology, with only classes A–C and F containing mammalian receptors (Hauser *et al.* 2017). Class A, the rhodopsin-like receptors, contains >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 that 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, and sensory and orphan receptors (Fig. 2A; Table S1, Supporting Information; King *et al.* 1990; Bass *et al.* 1996; Kokkola *et al.* 1998; Baranski *et al.* 1999; Campbell *et al.* 1999; Brown *et al.* 2000; Chambers *et al.* 2000; Erlenbach *et al.* 2001a; Zhang *et al.* 2002; Arias *et al.* 2003; Brown *et al.* 2011; Huang *et al.* 2015; Mukherjee, Bhattacharyya and Peralta-Yahya 2015; Liu, van Veldhoven and IJzerman 2016; Scott *et al.* 2019a). Additionally, a few GPCRs of class B1 have been functionally expressed (Fig. 2A; Kajkowski *et al.* 1997; Miret *et al.* 2002; Ladds *et al.* 2003).

While engineering of all these receptors has the coupling of a heterologous GPCR to the endogenous yeast mating pathway in common, a large 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 hotspots for rationalising and streamlining GPCR signalling carried out over the last few decades. The content of sections from 'Engineered G protein-coupling' to 'Evolution of GPCRs' is structured according to the schematic outline shown in Fig. 1B.

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 Gα, Gβ and Gγ (Syrovatkina *et al.* 2016). Exchange of GPCR-bound GDP to GTP activates the G protein, promoting the dissociation of the G protein from its receptor, and the separation of the Gα subunit

from the Gβγ 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 a connecting entity, the G protein plays a crucial role as a mediator between the heterologous receptor and the rest of the yeast mating pathway.

Generally, heterologous GPCRs show coupling preferences according to their native Gα coupling. In humans, the genome encodes 16 Gα subunits, which fall into four major classes, namely $G_{\alpha i/o}$, $G_{\alpha s}$, $G_{\alpha q}$ and $G_{12/13}$ (Syrovatkina *et al.* 2016; UniProt Consortium 2019). While coupling of heterologous GPCRs has been achieved using the endogenous yeast Gpa1 Gα subunit (Mukherjee, Bhattacharyya and Peralta-Yahya 2015), or the Gα heterologous counterpart, chimeric Gα subunits often couple more efficiently and are commonly used (Andrew J. Brown *et al.* 2000; Erlenbach *et al.* 2001a). Chimeric Gα subunits typically consist of the native Gα protein with C-terminal replacement of five 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 human receptors with seven chimeric Gα subunits from three different Gα subtypes. For seven out of eight GPCRs tested, coupling efficiency and ligand sensitivity improved when using chimeras (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:Gα protein couples, optimising the expression of G protein-encoding genes is an important parameter for successful GPCR signalling engineering. Indeed, optimally balanced levels for Gα, Gβ and Gγ, predicted using computational models (Bush *et al.* 2016; Bridge *et al.* 2018) 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 Gα has also been shown to be able to drastically affect agonist-sensing ability of olfactory receptors (ORs) when expressed in yeast cells (Minic *et al.* 2005; Fukutani *et al.* 2012).

Streamlining GPCR signalling

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 signalling, and may even negatively affect functional outputs, elimination of certain GPCR signalling 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; Erlenbach *et al.* 2001b; Leplatois *et al.* 2001; Mukherjee, Bhattacharyya and Peralta-Yahya 2015; Scott *et al.* 2019a).

More recently, Billerbeck *et al.* (2018) extended the minimising of the pheromone response by also deleting *BAR1* encoding a protease-cleaving α-factor into two inactive fragments excluding 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 minimised and tunable 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 signalling (*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).

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 (Hamilton, Watanabe and de Boer 1987; Hashi *et al.* 2018). 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 (Erlenbach *et al.* 2001b; O'Malley *et al.* 2009; Weston *et al.* 2015). Often however, the reason for poor expression of GPCR is left undefined, and as such the reason for failure of processing and cell surface localisation is unclear.

Especially affected by expression problems are olfactory receptors (Lu, Echeverri and Moyer 2003). This largely impacts OR deorphanisation studies, which is one of the reasons why currently only ~10% of the >400 human ORs have been deorphanised (Peterlin, Firestein and Rogers 2014; UniProt Consortium 2019). In yeast, ORs that have been successfully expressed include 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; Pajot-Augy *et al.* 2003; Minic *et al.* 2005; Tehseen *et al.* 2014; Yasi *et al.* 2019). 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).

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 signalling, are crucial for receptor cell surface expression and are responsible for negative regulation of GPCR signalling (Uddin *et al.* 2016). Early on, the functionalisation of the β2-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 was 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 (Ng, Brown and Walter 1996; O'Malley et al. 2009). 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 hypothesised by the activation of the unfolded protein response (O'Malley et al. 2009).

Fukutani et al. (2012) 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 localisation 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 led 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 et al. 2001b). 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).

Accessory proteins

Co-expression of odorant receptors with a receptor-transporting protein has been demonstrated to aid transport of both the eugenol-responsive OREG from mice and the human androstenone-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 GPRCs and thereby modulate their activity (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 (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 generalised, for a more in-depth overview of other accessory proteins, including guanine nucleotide exchange factors, guanine nucleotide dissociation inhibitors, GTPase-activating proteins and Gβγ-interacting proteins, the reader is referred to Sato et al. (2006) and Sato (2013).

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 >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 signalling in yeast (Trueheart, Boeke and Fink 1987; Trueheart and Fink 1989; Muller et al. 2003; 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 of native Ste12 transcription factor was combined with the full-sequence *LexA* bacterial repressor protein (Mukherjee, Bhattacharyya and Peralta-Yahya 2015; Shaw et al. 2019). 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 green fluorescent protein (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).

GPCR signalling 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; Ostrov et al. 2017; Shaw et al. 2019).

A HIS3 growth-coupled assay has been used for screening of weak partial agonists and inverse agonists (i.e. GPCR ligands shifting the equilibrium towards 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 (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 of the cytotoxic canavanine, only non-functional mutated receptors would survive supporting the analysis of functionally critical amino acids (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 signalling in yeast, the trend goes towards the use of novel, yeast-optimised fluorescent markers with high sensitivity and high signal-to-noise ratios (Nakamura, Ishii and Kondo 2013; Kaishima *et al*. 2016; Hashi *et al*. 2018).

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 allow for directed evolution of GPCRs with optimised expression levels, improved ligand sensitivity and altered ligand specificities (Schütz *et al*. 2016; Adeniran *et al*. 2018).

In one of the earliest studies 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 recognise 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, Rogan and Roth (2010) 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. Obviously, evolution-guided engineering of new ligand specificities has not shied away from medical research interest. As such, Ste2 underwent a substrate walking approach involving chimeric intermediate ligands to specifically recognise 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 (TRECs) 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.

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 (Hirz *et al*. 2013; Morioka *et al*. 2013; Routledge *et al*. 2016). 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.

GPCR STUDIES IN YEAST FOR BIOTECHNOLOGICAL AND MEDICAL APPLICATIONS

While traditionally yeast has played a major role in expression of GPCRs for crystallisation studies and mutagenesis studies for sequence-function explorations (Byrne 2015), this section will discuss some of the more recent demonstrations focussing 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).

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 analogues with an L-Cys2–L-Cys7 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 (FPRL1), six novel peptide agonists from a random plasmid library were identified, and the screening of a pyrrolopyrimidines library against two adenosine receptors yielded two novel antagonists (Fig. 3A; Klein *et al*. 1998; Campbell *et al*. 1999). Studies by Brown *et al*. (2003) showed that propionate and further short-chain carboxylic acids have agonistic effects on at-that-time orphan receptors GPR41 and GPR43.

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
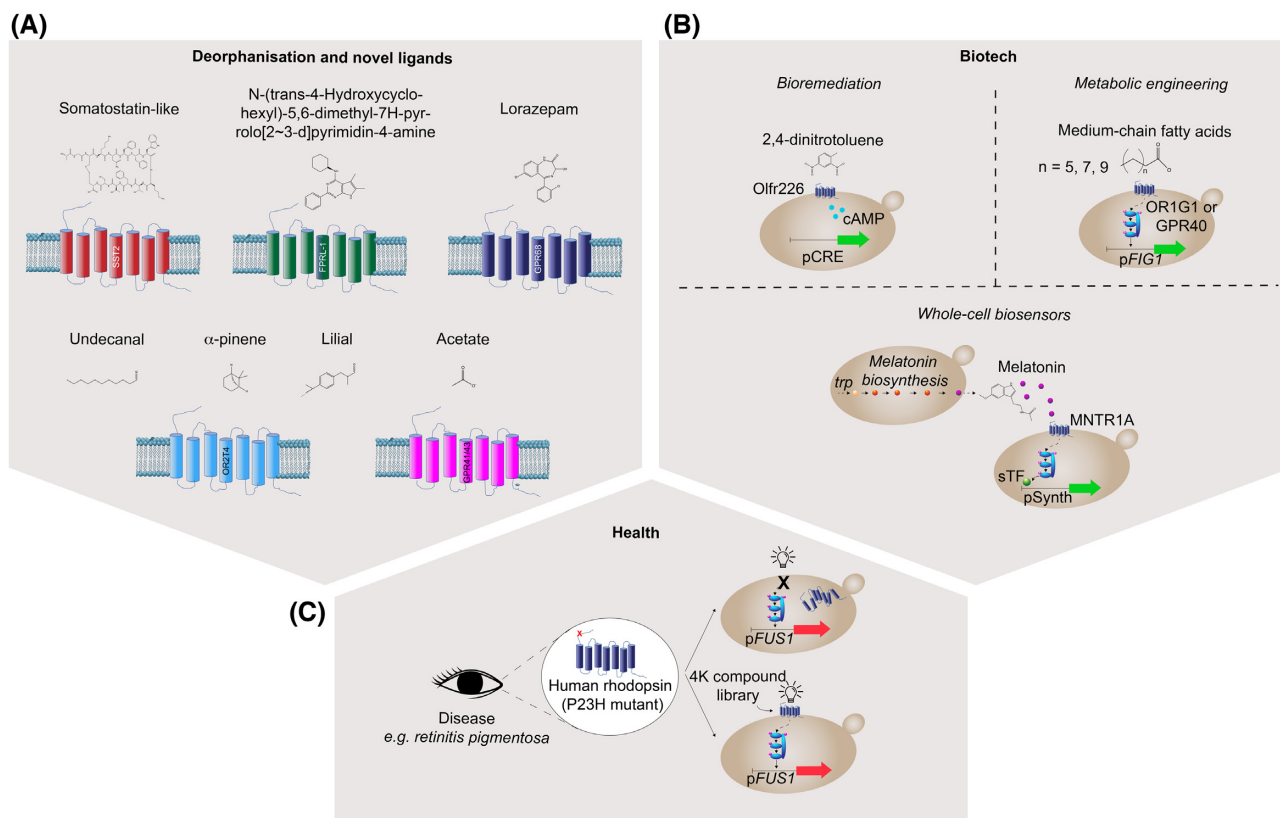
Figure 3. Applications using yeast for GPCR studies. **(A)** Examples in which yeast is used as a key enabling technology for GPCR deorphanisation studies and for identification of novel ligands. **(B)** Within biotechnology, functional expression of GPCRs in yeast has enabled the development of GPCR-based 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.

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 was found (Brown 2007; Overton, Fyfe and Reynet 2008). 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 ~400 ORs have been deorphanised (Fig. 2A and B; Flegel *et al*. 2013), and 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*. (2019) expressed seven human ORs in yeast. 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, emphasising the value of yeast-based screening systems 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 non-aromatic hydrophobic residue and an aromatic residue at positions 2, 4, 6, 11, 12 and 13, respectively (Manfredi *et al*. 1996).

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 optimisation (Jakočiūnas *et al*. 2015; Tong *et al*. 2015). However, the majority of products

synthetised in yeast that 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; Skjoedt *et al.* 2016; Taylor *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. 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 optimisation (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- to 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 *et al.* 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).

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 gramineaum, 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 the section 'Evolution of GPCRs', 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 *et al.* 2019a). 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-stabilising compounds with the ultimate goal of developing a drug to combat vision loss and to ameliorate disease (Scott *et al.* 2019b). Although 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 *et al.* 2019a,b).

OUTLOOK

As reviewed herein, >50 GPCRs have been functionally expressed in yeast (Fig. 2; Table S1, Supporting Information; Shaw *et al.* 2019). As guiding principles, for functional coupling of heterologous GPCRs, N-terminal receptor fusions with signalling sequences have been shown to enhance the expression of several receptors, and Gα coupling specificity has 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 have 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 (Mukherjee, Bhattacharyya and Peralta-Yahya 2015; Shaw *et al.* 2019). 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 minimised 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 signalling has underscored yeast as a valuable resource for functional GPCR studies (Mukherjee, Bhattacharyya and Peralta-Yahya 2015; Ehrenworth, Claiborne and Peralta-Yahya 2017; Ostrov *et al.* 2017; Adeniran *et al.* 2018; 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; Adeniran *et al.* 2018; Scott *et al.* 2019b; Shaw *et al.* 2019). Beyond this, the means to study GPCR signalling 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 (Scott *et al.* 2019b; Yasi *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 (Jarque, Bittner and Hilscherová 2016; Ostrov *et al.* 2017), 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 (Mukherjee, Bhattacharyya and Peralta-Yahya 2015; Scott *et al.* 2019b), 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 (Siddiqui *et al.* 2012; Galanie *et al.* 2015; Nielsen and Keasling 2016; Luo *et al.* 2019). Metabolic engineering of bioactive natural products often includes balancing gene expression of biosynthetic pathways, expansive pathway optimisation based on homology searches, codon-optimisations and compartmentalisation strategies (Brown *et al.* 2015; Galanie *et al.* 2015; Luo *et al.* 2019). Taken together, this makes up a vast multiparametric 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 minimise 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 (Runguphan, Qu and O'Connor 2010; Moses *et al.* 2014), and derivatised substrates in yeast (McCoy and O'Connor 2006; Li *et al.* 2018), development and application of GPCR-based biosensors are 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 towards human illnesses, these studies should also help establish more broadly applicable expression guidelines to further advance GPCR signalling studies in general.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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