Biosensors Design in Yeast and Applications in Metabolic Engineering

Chenxi Qiu, Haotian Zhai, Jin Hou*

State Key Laboratory of Microbial Technology, Shandong University, Qingdao, 266237, P. R. China

* Corresponding author: Prof. Jin Hou, State Key Laboratory of Microbial Technology, Shandong University, Binhai Road 72, Qingdao, Shandong, 266237, P. R. China; email: houjin@sdu.edu.cn.

Abstract:

Engineering microbial cell factories is a potential approach of sustainable production of chemicals, fuels and pharmaceuticals. However, testing the production of molecules in high throughput is still a time-consuming and laborious process since product synthesis usually does not confer a clear phenotype. Therefore, it is necessary to develop new techniques for fast high-producer screening. Genetically encoded biosensors are considered to be promising devices for high-throughput analysis owing to their ability to sense metabolites and couple detection to an actuator, thereby facilitating the rapid detection of small molecules at single-cell level. Here, we review recent advances in the design and engineering of biosensors in *Saccharomyces cerevisiae*, and their applications in metabolic engineering. Three types of biosensor are introduced in this review: transcription factor based, RNA-based, and enzyme-coupled biosensors. The studies to improve the features of biosensors are also described. Moreover, we summarized their metabolic engineering applications in dynamic regulation and high producer selection. Current challenges in biosensor design and future perspectives on sensor applications are also discussed.

Key words: Biosensors, Metabolic engineering, High-throughput screening, Dynamic regulation

Introduction

Bio-based production of chemicals using renewable feedstocks is considered a possible replacement for current production from petrochemicals. In past decades, a wide variety of valuable products, including biofuels, bulk chemicals, fine chemicals, nutraceuticals and pharmaceuticals, has been produced in a bio-based manner using microbial cell factories. Many attempts are often required to rewire the

metabolism of organisms to establish microbial cell factories that can efficiently convert a renewable feedstock into a target compound. The advancement of genome-editing tools such as CRISPR-Cas9 has significantly speeded up the engineering of microbial cell factories. Engineering approaches such as introduction of heterologous key enzymes of metabolic pathways, optimization of endogenous metabolism, and engineering of cofactor and energy metabolism have been widely used to improve yields, titers and productivity (Keasling, 2010, Woolston *et al.*, 2013). Aside from rational engineering, approaches like mutagenesis, evolution or multiplex genome engineering, which require high-throughput screening, are also essential to construct microbial cell factories. However, evaluation of microbial phenotypes and product synthesis by traditional analytical methods such as chromatography and mass spectrometry is slow and cumbersome, and is thus a major rate limiting step in metabolic engineering. Hence, it is critical to develop rapid analysis tools such as biosensors offering the potential for strain evaluation and high-throughput screening that can accelerate the classic design-build-test-learn cycle (Dietrich *et al.*, 2013, Li *et al.*, 2015).

Another challenge of metabolic engineering is that the introduction of heterologous pathways often results in metabolic imbalance and the accumulation of toxic intermediary metabolites, which hamper the achievement of high yields, titers and productivity of desired chemicals. Excessive expression of the genes in a certain pathway will waste resources and increase the burden on cells. It may also drain precursor metabolites or accumulate intermediates to toxic levels, thereby causing metabolic imbalance. In these cases, dynamic control and regulation of the metabolic pathway is a potential approach to address the problem. This approach also requires biosensors, to sense the change in metabolite concentrations and dynamically regulate gene expression.

Genetically encoded biosensors can detect either environmental signals (e.g. temperature, pH, metabolites, or proteins) or intracellular metabolites. They sense the signals and couple them to an actuator output (e.g., fluorescent reporters, regulatory switches, or antibiotic resistance). Biosensors have proven to be valuable tools to sense various metabolites and regulate metabolism, and have been used for high-producer screening or evolution. Although many recently reported biosensors and applications have focused on prokaryotic hosts, examples of biosensor development in eukaryotic cells are increasing. In this review, we introduce biosensor design and metabolic engineering applications in yeast. Three types of biosensor-transcription factor (TF) based, RNA-based, and enzyme-coupled biosensors are

introduced. Engineering approaches to improve their performance are also described. Their applications in metabolic engineering, including dynamic pathway regulation and high-throughput screening are discussed. Finally, we discuss current limitations of, and future perspectives on, biosensors.

TF-based biosensors

TF-based biosensors can respond to a change in intracellular or extracellular ligands and hence alter the transcription of genes. These biosensors can be constructed by introducing heterologous transcription activators/repressors or exploring endogenous yeast TFs and promoters to sense intracellular metabolites, extracellular ligands or environmental change. Aside from that, we also introduced the biosensors which can sense the ligands through signal transduction pathways such as G-protein-coupled receptors (GPCRs). The biosensors that sense the environmental changes to regulate transcription are also described.

Introduction of heterologous transcription factors (TFs) for functional biosensors

TFs are proteins that can respond to a ligand or environmental change, which affects the binding of DNA and hence changes the transcription of a downstream gene(s). The most common structural characteristics of TFs are the ligand-binding domain (LBD) and the DNA-binding domain (DBD). In recent years, the development, characterization and application of TF-based biosensors in prokaryotes has aroused significant interest. Compared with eukaryotes, transcriptional regulation in prokaryotes is relatively simple. In prokaryotes, TFs often bind a ligand directly, and then undergo an allosteric conformational change that affects their DNA binding affinity to activate or repress gene transcription. Such TF-based biosensors can be divided into transcriptional activators and transcriptional repressors. Some TF-based biosensors from prokaryotes have been proven to be functional in eukaryotes (Li *et al.*, 2015, Skjoedt *et al.*, 2016, Wang *et al.*, 2016). The prokaryotic promoters cannot be used directly in eukaryotes. Instead, the operator sequence of a TF has been inserted under the control of the eukaryotic promoter and the TF was then expressed to create a biosensor that responded to a specific ligand. Examples of such biosensors are listed in Table 1.

The group of transcriptional factors which can sense the ligand, leads to allosteric conformational change, then bind to or dissociate from the operator sequences in prokaryotes has been widely applied in eukaryotes (Gari *et al.*, 1997). For example, transcriptional repressor FapR from *Bacillus subtilis*is

typically located adjacent to a fatty acid synthesis operon, whose expression is driven by a hybrid promoter possessing a cis-regulatory fapO-operator. It can bind intracellular malonyl-CoA, cause a conformational change and relieves from fapO-operator to activate the expression of the fatty acid synthesis genes (Li *et al.*, 2015). It has been successfully transferred to yeast to construct the malony-CoA sensor.

When transferring the prokaryotic TF to yeast, its operator needs to insert into the promoter. Yeast promoters generally contain a core promoter and upstream activating sequences (UAS). Placing the operators in different positions relative to the promoter in yeast will have different activation or inhibition effects. When placing the operator into the core promoter, the TFs regulate gene transcription in a ligand-dependent manner through binding to the operator site and sterically preventing transcription (Fig. 1a). For instance, *B. subtilis* transcriptional repressor FapR and its cognate DNA binding site fapO have been engineered to construct a malonyl-CoA sensor in *Saccharomyces cerevisiae* (Li *et al.*, 2015). Bacterial FadR transcriptional repressors were used to construct fatty acid/fatty acyl-CoA biosensors in *S. cerevisiae* (Wei *et al.*, 2014, Dabirian *et al.*, 2019). Similar biosensors to sense molecules such as xylose, camphor, and doxycycline have also been developed (Table 1).

Prokaryotic TFs can also be used in transcriptional activators in eukaryotic cells. When the TF is fused with an activation domain (AD) for transcription, it can activate gene transcription in a ligand-dependent manner (Fig. 1b). In this case, the operator site is often inserted into the upstream activation region. ADs such as the VP16 AD(s) from herpes simplex virus or the Gal4 transactivation domain (Gal4 AD) are often fused with the TF (Gari *et al.*, 1997, Ellis & Wolfgang, 2012, Siedler *et al.*, 2014, Feng *et al.*, 2015). For instance, a tetracycline or doxycycline regulation system was constructed in which TetR was converted from a repressor to an activator by fusing a VP16 AD to the C-terminus of TetR (Gossen & Bujard, 1992). By fusing the N-terminal domain of Ada protein, which detects methylating compounds, to the Gal4 AD, Moser et al. built a functional sensor for methyl phosphotriester adducts in yeast (Moser *et al.*, 2013). When fused with the transcriptional activator VP16, the transcriptional repressor FapR was converted to a transcriptional activator in living mammalian cells. On increase of malonyl-CoA, FapR underwent conformational change and dissociated from the promoter, thereby resulting in a decrease of transcription levels (Ellis & Wolfgang, 2012).

Compared with prokaryotic transcriptional repressors, there have been relatively few studies transplanting prokaryotic transcriptional activators into eukaryotes. However, one successful example was when Skjoedt *et al.* developed biosensors in *S. cerevisiae* based on transcriptional activators from the prokaryote superfamily of LysR-type transcriptional regulators (Skjoedt *et al.*, 2016). Unlike transcriptional repressors, there are three potential binding sites of LysR-type transcriptional regulators. For example, the BenM tetramer from *Acinetobacter sp.* ADP1 binds to two sites and blocks the binding of RNA polymerase in the absence of cis,cis-muconic acid (CCM) (Fig. 1c). In the presence of CCM, BenM undergoes a conformational change, which changes the binding site to facilitate access of RNA polymerase thus activating transcription (Skjoedt *et al.*, 2016). Skjoedt *et al.* employed BenM and the operator in yeast to construct CCM-responsive biosensors. Biosensors that responded to naringenin, muonic acid (Skjoedt *et al.*, 2016) and itaconic acid (Hanko *et al.*, 2018) were also constructed. The different mode of activators compared with that of repressors and the inherent need for more extensive engineering, clearly demonstrated why prokaryotic transcriptional activators are more difficult to transplant into eukaryotes.

To obtain a biosensor that is suitable for application, features such as specificity, sensitivity and dynamic range often need to be optimized. The properties of TFs, promoters, operator position and numbers have been showed to affect these features (Mahr & Frunzke, 2016). The insertion position and number of operator sites is often optimized to change the dynamic and operational range of a biosensor (Blake *et al.*, 2003, Khalil *et al.*, 2012). For instance, Wang *et al.* showed that inserting the operator immediately upstream of the TATA-box resulted in an obvious dose-response curve in xylose-sensing/regulation gene circuits (Wang *et al.*, 2016). In malonyl-CoA sensor design, Li *et al.* placed one operator immediately upstream of the TATA box of the *GPM1* promoter and obtained threefold repression of transcription. On the contrary, when placing one operator in the same position relative to the $TEF1_{UAS}$-$GAL1_{core}$ synthetic promoter, transcription was not repressed by *fapR* expression, but inserting one operator 7 bp downstream of the TATA box in the $TEF1_{UAS}$-$GAL1_{core}$ synthetic promoter could result in 20-fold repression of transcription. In a TATA-less promoter such as the *TEF1* promoter, inserting three *fapO* operators in the promoter was essential to obtain a repression effect. Therefore, it is necessary to consider the promoter characteristics to optimize the dynamic output range and the operational range of a biosensor. In a recent study, Dabirian & Li evaluated the effect of positions and numbers of *fapO* sites in

different promoters, and showed that the insertion of up to four *fapO* sites downstream of the TATA box in the *CCW12* promoter caused the dynamic range of transcription to change by up to 95-fold (Dabirian & Li, 2019). The expression level of a repressor is another factor that might influence the expression of a gene (Wang *et al.*, 2016). Wei *et al.* proved that FadR under the control of the *TEF1* promoter had a better repression effect than *CYC1* promoter-controlled expression (Wei *et al.*, 2014). Another method to enhance transcriptional inhibition is the fusion of a transcriptional repressor and a heterologous TF. For example, on fusion with a transcriptional repressor such as Mig1 or Rox1, the repression effect of FapR can be stronger than that of the construct only expressing FapR (Dabirian & Li, 2019). A nuclear location signal (NLS) is also essential in eukaryotes to transport TFs to the nucleus. Li *et al.* showed that the absence of a NLS could lead to low repression effect of FapR (Li *et al.*, 2015).

Evolution of TFs is an important method to change the specificities and improve the features of biosensors. The naturally existing TFs are limited, even with database mining, and many compounds of interest still cannot be correlated with a TF. Therefore, it is necessary to evolve existing TFs with new ligand specificity. However, most studies of direct evolution of TFs are performed in bacteria and only a few TFs have been evolved in yeast. For instance, Skjoedt *et al.* demonstrated that directed evolution of the LBD of BenM enabled the identification of TF variants with increased output in the presence of CCM (Skjoedt *et al.*, 2016). Recently, the same group reported that from a single round of directed evolution of the LBD of BenM, it was possible to select TF variants with changed ligand specificity, increased dynamic output range, shifts in operational range, and a complete inversion of function from activation to repression (Snoek *et al.*, 2019). These studies demonstrate the potential to evolve biosensors directly in eukaryotes.

Some synthetic biosensors are not based on the expression of prokaryotic TFs in eukaryotic cells. Instead, the DBD or AD in specific TFs and specific LBDs were used as modules for new biosensor design. For example, an isopentenyl diphosphate (IPP)-sensing biosensor was created based on the ability of IPP isomerase to dimerize on binding IPP. Two chimeric proteins were constructed: one was a chimera of IPP isomerase and the AD of TF Gal4, and the other was the DBD of TF Gal4 and IPP isomerase. Upon IPP binding, IPP isomerase dimerization can bring the AD and DBD in close enough proximity to activate transcription from a *GAL* promoter, thereby reflecting the IPP level. The IPP isomerase was also replaced by Erg20 to improve the dynamic range of the sensor. In another study, a

conditionally stable LBD was used to design the biosensor. The sensor was constructed by fusing a LBD with a fluorescent protein or a transcriptional activator. In the absence of ligand, conditionally stable LBDs are degraded. Binding of the ligand stabilizes the LBD and prevents its degradation, leading to enhanced reporter activity. Based on this principle, biosensors to sense digoxin and progesterone were developed in yeast and mammalian cells (Feng *et al.*, 2015).

Based on such designs, it is possible to integrate different signals together to construct logic gates (Lutz & Bujard, 1997, Bikard *et al.*, 2013). Mazumder & McMillen established a dual-mode promoter through integration of activation and repression signals (Mazumder & McMillen, 2014). They incorporated five steroid hormone response elements and a lac operator upstream and downstream of the TATA box, respectively, of a yeast minimal *CYC1* promoter, allowing activation through the testosterone responsive androgen receptor and inhibition through the LacI repressor (Mazumder & McMillen, 2014). In addition, AND-gate dynamic controllers were constructed by combining two dynamic control strategies, containing inducible promoters and sensing-regulation (Teo & Chang, 2014). Inducible promoter enhancer sequences (*CUP1* and *PHO5*) were fused to a synthetic *GAL1* core promoter containing a fatty acyl-CoA operator; thereby two inputs (fatty acid and copper presence/phosphate starvation) were required to switch the AND gate ON. Such controllers will be useful for synthetic biology applications in which multiple signals must be integrated to give a single output.

Exploring yeast endogenous TFs and promoters as biosensors

In addition to introduction of heterologous TFs to create biosensors in yeast, it is also possible to explore endogenous yeast TFs and promoters as biosensors. For example, Zhang *et al.* used a native oxidative stress response TF Yap1 to construct a NADPH/$NADP^+$ redox biosensor in *S. cerevisiae* (Zhang *et al.*, 2016). Yap1 is oxidized upon oxidative stress and activates the expression of genes involved in oxidative stress defense, including the biosynthesis genes for glutathione and thioredoxin, and their reductases. Oxidation of Yap1p can be reversed by reduced thioredoxin using electrons from cytosolic NADPH. The NADPH/$NADP^+$ redox state thus indirectly regulates the equilibrium between oxidized and reduced Yap1, and the transcription of Yap1 regulated genes can reflect the NADPH/$NADP^+$ redox state. Endogenous regulation systems can also be applied for dynamic control. For example, Yuan & Ching identified several ergosterol-responsive promoters for dynamic control of *ERG9* expression. This

dynamic control improved the production of isoprenoids such as amorpha-4,11-diene (Yuan & Ching, 2015).

When no natural protein or RNA can be used as a biosensor, high-throughput genomics technologies can be applied to identify and build novel biosensors. In *Escherichia coli*, whole-genome transcriptome analysis has been performed to identify promoters that respond to farnesyl pyrophosphate (FPP), and these promotors were then used to control accumulation of FPP (Dahl *et al.*, 2013). Similarly, a yeast 1-butanol biosensor was identified through genome-wide transcriptome analysis. Two promoters that responded specifically to 1-butanol were used to distinguish differences in production levels among different 1-butanol producer strains (Shi *et al.*, 2017). This provides a universal methodology for the discovery of biosensors sensitive to particular stresses or metabolites.

GPCRs engineering for biosensor design

Unlike prokaryotic TFs, eukaryotic TFs rarely interact directly with RNA polymerase. In addition, eukaryotic TFs do not usually bind ligands directly; instead, the ligands bind to receptors, then the signal passes to the TF that directly controls gene expression via a signal transduction pathway. Among the signal transduction systems, GPCRs are the largest and the most versatile superfamily. A large class of yeast-based biosensors is dependent upon GPCRs. The GPCR is coupled to a G-protein composed of Gα, Gβ, and Gγ subunits. When the GPCR binds a ligand, which induces a conformational rearrangement of the receptor, activating the heterotrimeric G proteins, resulting in the dissociation of Gα from the heterodimeric β- and γ-subunits (Gβγ), which activates the downstream signaling pathway. GPCRs are able to detect a diverse range of molecules, including proteins, photons, ions, and small molecules (Fig. 2a) (Pierce & Lefkowitz, 2001). It should be noted that, unlike the abovementioned TF-based biosensors, GPCRs usually detect extracellular ligands, not intracellular metabolites.

Glucose sensing and the pheromone response pathway are the two native GPCR signaling pathways in *S. cerevisiae* (Versele *et al.*, 2001). The pheromone-mating pathway is normally used by haploid cells to detect potential mating partners. It has been engineered to construct a quorum sensing system (Williams *et al.*, 2013). In this system, populations consisting of only mating type “a” cells produce and respond to extracellular α-type pheromone, which controls gene expression in a population-density-dependent manner. Positive feedback dynamics were tuned by using different pheromone-responsive promoters as

well as different versions of pheromone genes. The authors also constructed a second system, which controlled α-pheromone expression via the aromatic amino acid-responsive *ARO9* promoter. The pheromone-mediated signal transduction pathway was also coupled with heterologous GPCRs to response to new ligands (Fig. 2a). For example, Mukherjee *et al.* used GPCRs known to bind medium-chain fatty acids in mammalian cells to construct chemical sensors in yeast (Mukherjee *et al.*, 2015). Introduction of olfactory receptor OR1G (Sanz *et al.*, 2005) and the free fatty acid receptor GPR40 (Itoh *et al.*, 2003) enabled yeast to detect C8-C12 fatty acids. Introduction of a synthetic response unit improved both the dynamic and linear range of the GPCR OR1G1-based sensors (Mukherjee *et al.*, 2015). In following study, this group constructed a serotonin sensor by swapping OR1G1 for a GPCR known to bind serotonin (Ehrenworth *et al.*, 2017). After testing several serotonin GPCRs, 5-HT4 GPCR had the best performance. In these designs, it was necessary to delete endogenous yeast genes to avoid cell cycle arrest (FAR1) and nonspecific activation (STE2), and to reduce the spontaneous rate of GPCR inactivation upon chemical sensing (SST2).

Recently, an insulated, modular GPCR signal transduction system was constructed in yeast (Mukherjee *et al.*, 2015, Ehrenworth *et al.*, 2017). A model strain retaining only the core signaling elements was built by removing nonessential components, native transcriptional feedback regulation, and all connections to the mating response. Using five different GPCRs, the generated biosensors could respond to desired concentrations of peptides, metabolites, and hormones relevant to human health. This work provides a framework to reprogram GPCR-based signaling sensing systems.

Biosensors sensing environmental change

Changes in the environment, such as pH, light and temperature, are very important factors affecting the growth and metabolism of microorganisms. Therefore, development of biosensors to respond to environmental signals can provide us with new opportunities to control metabolism.

Zhou *et al.* introduced optogenetic tools using light-switchable transcription modules to control gene expression in yeast (Fig. 2b) (Zhao *et al.*, 2018). Two optogenetic circuits, OptoEXP and OptoINVRT, were designed based on a blue light-activated gene expression system. The optogenetic circuits were designed to shift cells from a light-induced growth phase to a darkness-induced production phase. A temperature-dependent control system was developed based on a modified GAL regulatory system

(Zhou *et al.*, 2019). A temperature-sensitive Gal4 mutant Gal4M9 was created by directed evolution, and used to control the expression of *GAL* promoter-driven pathway genes.

The quorum sensing system, which can sense cell density to control gene expression, is the most frequently used system for dynamic control of cell growth and product synthesis. Although it has been widely studied in bacteria, only two synthetic quorum sensing circuits have been reported in *S. cerevisiae.* One is the abovementioned GPCR mating-type-specific pheromone-mediated quorum sensing circuit (Williams *et al.*, 2013). The other is a hormone cytokinin-mediated positive feedback circuit (Chen & Weiss, 2005). *Arabidopsis thaliana* cytokinin signal synthesis and receptor components were integrated with yeast endogenous protein phosphorylation elements and response promoters. Under positive-feedback regulation, the cells synthesize cytokinin, which diffuses into the environment and activates a hybrid phosphorylation-signaling pathway, thereby inducing population density-dependent gene expression.

Recently, a pH sensing promoter was developed (Rajkumar *et al.*, 2016), demonstrating a general strategy for designing and engineering synthetic yeast promoters that are inducible by environmental conditions or stresses. Using TF binding data, Rajkumar *et al.* selected the *YGP1* promoter as a candidate promoter, and then modified the TF binding sites in its upstream activation sequence to improve low-pH performance. This pH-sensing promoter could be used to increase lactic acid production (Rajkumar *et al.*, 2016).

RNA-based biosensors

RNA-based biosensors such as riboswitches or ligand-dependent ribozymes are also extensively present in organisms, especially prokaryotes. Compared with TF-based biosensors, RNA-based biosensors offer faster responses since the RNA has already been transcribed and is therefore readily available for ligand binding. A riboswitch is a regulatory segment of some mRNAs within the 5′-untranslated region that can selectively bind to a ligand and consequently change the secondary structure of the RNA, thereby regulating transcription termination, translation initiation, or the rate of mRNA decay (Link & Breaker, 2009). Riboswitches selectively bind to target metabolites via an aptamer domain (ligand-binding domain). Synthetic aptamers that can bind new ligands have been obtained by creating synthetic aptamer libraries and selecting the RNAs in vitro (Mairal *et al.*, 2008). However, because of the different

translation mechanisms in prokaryotes and eukaryotes, riboswitches are hard to apply in eukaryotic systems. An approach that can be relatively easily implemented in eukaryotic systems is to use ribozyme-based riboswitches in which the binding of ligands induces self-cleavage activity and destabilizes the mRNA; in this way, the translation of the mRNA is controlled in a ligand dependent manner.

Compared with TF-based biosensors, only a restricted number of RNA aptamers have been constructed in yeast (Table 1; Fig. 2c). Michener *et al.* employed a theophylline responsive ribozyme to control the expression of GFP as a biosensor in yeast. Using such a sensor, a caffeine demethylase library was iteratively screened (Michener & Smolke, 2012). Lee *et al.* constructed a synthetic riboswitch, using a self-cleaving ribozyme *glmS* that integrated into the 3′-untranslated region of the gene encoding cytosine deaminase to cleave its own transcript in response to the intracellular glucosamine 6-phosphate (GlcN6P) concentration (Lee & Oh, 2015). The conversion of fluorocytosine to fluorouracil by cytosine deaminase is generally suicidal because it leads to dysfunction of RNA. Thereby, a suicide riboswitch was developed for evolutionary engineering. In addition, development of synthetic ligand-dependent ribozymes is an effective approach to construct new biosensors. Klauser *et al.* reported a novel hammerhead ribozyme-based artificial riboswitch that affects post-transcriptional regulation of gene expression (Klauser *et al.*, 2015). Meanwhile, they (Klauser *et al.*, 2015) attached a synthetic neomycin aptamer to the catalytic core of the type III hammerhead ribozyme and constructed a neomycin-dependent ribozyme to control gene expression in yeast.

Enzyme-coupled biosensors

Using enzyme-catalyzed reactions that convert a target metabolite into a detectable chromogenic or fluorogenic compound is another approach to construct biosensors. This requires exploration of specific enzymes to catalyze conversion of the target metabolite into the chromogenic or fluorogenic compound. This strategy has been successfully applied in yeast for high throughput screening. For instances, DeLoache *et al.* demonstrated a one-step enzyme coupled reaction of the benzylisoquinoline alkaloid (BIA) pathway intermediate L-3,4-dihydroxyphenylalanine (L-DOPA) to screen yeast-active tyrosine hydroxylases (DeLoache *et al.*, 2015). This enzyme-coupled biosensor takes advantage of a plant DOPA dioxygenase that converts L-DOPA into the yellow fluorescent pigment betaxanthin (Gandia-Herrero *et*

al., 2005). Using this biosensor, the activity of tyrosine hydroxylase was improved through and screening, thus increasing L-DOPA titer (DeLoache *et al.*, 2015). This biosensor enabled (S)-reticuline production in yeast, which is the key intermediate in synthesis of BIAs. In another study, Lin *et al.* developed a coupled enzyme assay that converts AATase-produced CoA-SH to succinyl-CoA by α-ketoglutarate dehydrogenase (α-KGDH) (Lin *et al.*, 2016). The coupled α-KGDH reaction reduces NAD^+ to NADH, enabling spectrophotometric measurement of AATase activity for high throughput screening of ester synthesis.

Recently, a simple and robust enzyme-coupled malonyl-CoA biosensor was developed by repurposing 1,3,6,8-tetrahydroxynaphthalene synthase (THNS; also referred to as RppA), a type III polyketide synthase (PKS) to 1,3,6,8-tetrahydroxynaphthalene (THN), which is then spontaneously converted to red-colored flaviolin (Yang *et al.*, 2018). The RppA biosensor was applied to screen knockdown gene targets capable of accumulating malonyl-CoA, resulting in enhanced production of 6-methylsalicylic acid, aloesone, resveratrol and naringenin in *E. coli*. The application has not yet been demonstrated in yeast.

Metabolic engineering applications of biosensors

Biosensors have been widely applied in metabolic engineering and largely facilitate pathway dynamic control and high producers selection (Fig. 3a and b). For high producers selection, both high-throughput screening using fluorescence-activated cell sorting (FACS) or color-based selection and adaptive laboratory evolution (ALE) are frequently used strategies. We will introduce recent applications in yeast.

Dynamic pathway regulation

In microorganisms, metabolite biosynthesis is usually controlled tightly by the complex metabolic regulatory network to avoid the accumulation of toxic intermediates and rapidly adapt to environmental change. However, high expression of heterologous biosynthetic pathways or excessive inhibition/activation of an endogenous pathway through metabolic engineering may result in the imbalance of metabolic flux and affect cell growth, thereby having a deleterious influence on titers, yields, and productivities of target materials. To engineer efficient microbial cell factories, it is

therefore important to control endogenous and heterologous pathways to appropriate levels at specific time points. Dynamic control of pathways will allow the microorganism to change the metabolic flux distribution depending on the intracellular or environmental conditions in real time.

In yeast, most examples of dynamic control focus on separating the cell growth phase from the target product production phase (Fig. 3a). The most common method is to use the glucose-sensing *HXT1* promoter to achieve dynamic regulation of metabolic pathways. Expression is activated when glucose is present in the medium, but it is inhibited when the glucose concentration is low. For example, Scalcinati *et al.* used the *HXT1* promoter to control *ERG9* expression, shifting carbon flux from sterol synthesis to sesquiterpene α-santalene production, increasing the productivity by 3.4-fold (Scalcinati *et al.*, 2012). Zhao *et al.* explored the use of the *HXT1* promoter to dynamically control *ERG20* expression to redistribute precursor geranyl diphosphate flux. After optimizing glucose and ethanol ratios, monoterpene geraniol production increased (Zhao *et al.*, 2017).

Aside from glucose, there have been few studies of dynamic control of metabolic pathways using biosensors, which respond to intracellular metabolites in yeast. Yuan & Ching used the ergosterol response promoter to dynamically control *ERG9* expression. When ergosterol was in excess, the transcription of *ERG9* was downregulated (Yuan & Ching, 2015). This feedback operation allowed the sterol level to be maintained appropriately, significantly improving the amorpha-4,11-diene titer to around 350 mg/L.

Dynamic control can also be implemented at different levels. David *et al.* implemented a two-stage system to control the key pathway intermediate malonyl-CoA for 3-hydroxypropionic acid production (David *et al.*, 2016). At the upper level of the control system, the fatty acid synthase gene *FAS1* was expressed under the control of the glucose-sensitive *HXT1* promoter, ensuring the downregulation of fatty acid biosynthesis, which resulted in accumulation of malonyl-CoA in glucose limiting conditions. In the second level, a malonyl-CoA biosensor was used to activate the 3-hydroxypropionic acid production pathway in response to the malonyl-CoA level. Introduction of dual pathway control can achieve hierarchical control by both environmental conditions and intracellular metabolites.

In addition to response to intracellular products, there are also some examples in yeast of dynamic pathway regulation that senses extracellular environmental change, such as in light, temperature or cell

density. Light control systems are attractive for metabolic engineering applications. Zhao *et al.* introduced two optogenetic circuits to transfer cells from a light-induced growth phase to a dark-induced production phase, based on the blue light-activated *EL222* gene expression system (Zhao *et al.*, 2018). This system was constructed based on a bidirectional control afforded by combining OptoEXP and OptoINVRT circuits together. The expression of pyruvate decarboxylase was controlled by OptoEXP and acetolactate synthase or lactate dehydrogenase was controlled by OptoINVRT. Thereby, the metabolism could be transferred from ethanol production to lactic acid or isobutanol production by optogenetic control. Optogenetic control of engineered pathways enables periodic light pulses to tune expression of an enzyme that is necessary for cell growth during the production phase of fermentation, thus increasing yields of isobutanol and 2-methyl-1-butanol (Zhao *et al.*, 2018).

Zhou *et al.* developed a temperature-dependent dynamic control strategy to decouple cell growth and lycopene production (Zhou *et al.*, 2019). In the first stage, when the temperature was controlled at 30 °C, the strain maintained rapid growth, but the lycopene content was very low. In the second stage the culture temperature was changed to 24 °C to induce the production of lycopene. In this stage, the biomass only increased slightly, while lycopene accumulation increased significantly. Using the same strategy, this group introduced a Gal4M9-based temperature response regulation system to separate astaxanthin production from cell growth, producing 235 mg/L of (3S,3'S)-astaxanthin (Zhou *et al.*, 2019). Dynamic control sensing cell density is also an effective approach to separate cell growth from product synthesis. Williams *et al.* combined the quorum-sensing circuit with RNA interference (RNAi) module for achieving dynamic suppression of the metabolic pathway (Williams *et al.*, 2015). On reaching a high population density, the circuit autonomously switched on para-hydroxybenzoic acid (PHBA) biosynthesis and a triggered an RNAi module to control flux through the shikimate pathway, thereby increasing PHBA titer.

High producers selection

Due to the complexity of organisms and limited knowledge of mechanisms, strain engineering based on rational design often cannot achieve the desired productivity. Selection of high-producers based on the creation of mutant libraries and high-throughput screening is an effective strategy to overcome the limitations of rational engineering (Fig. 3b). The use of biosensors has proven to be very valuable for

library screening. The library can be a mutation library of the key enzyme in a metabolic pathway, or a genome-wide mutation library (Tang *et al.*, 2013, Siedler *et al.*, 2014, Cheng *et al.*, 2015, DeLoache & Russ, 2015, Lee & Oh, 2015).

When the actuator of biosensors is fluorescence or color, they are usually used for high-throughput screening based on FACS or color-based selection (Fig. 3b). For example, DeLoache *et al.* developed an enzyme-coupled biosensor for detecting L-DOPA in *S.cerevisiae* (DeLoache *et al.*, 2015) and used this sensor to screen a tyrosine hydroxylase mutant library. An active tyrosine hydroxylase was obtained and its expression improved L-DOPA production by 2.8-fold. When biosensors are coupled with cell growth, ALE can be performed for high-producer selection (Fig. 3b). Lee & Oh designed a synthetic suicide riboswitch to couple the intracellular glucosamine 6-phosphate (GlcN6P) concentration to cell growth. The *glmS* ribozyme was integrated into the 3'-untranslated region of *FCY1*, which encodes cytosine deaminase. This deaminase can convert 5-fluorocytosine to 5-fluorouracil, resulting in cell death due to RNA dysfunction under fluorocytosine addition (Lee & Oh, 2015). Growth of the strains was recovered as the GlcN6P level increased. Combining directed evolution with growth-based selection, an efficient glutamine-fructose-6-phosphate transaminase and haloacid dehalogenase-like phosphatase was screened and an N-acetyl glucosamine producer strain was isolated.

Besides enzyme selection, screening potential targets on a genome-wide scale is necessary to discover new mutations and new "design principles", that contribute to the development of strains with desired phenotypes. Genomic libraries are among the most frequently used libraries for screening. Based on a synthetic gene circuit including the S-adenosylmethionine (SAM)-responsive MetJ transcriptional repressor, a genomic library was screened to identify the *Gal11* gene as a novel multicopy enhancer of SAM level in *S. cerevisiae*. Li *et al.* developed a genetic sensor responsive to malonyl-CoA in *S. cerevisiae* and coupled it with a genome-wide overexpression library to enhance the intracellular concentration of malonyl-CoA (Li *et al.*, 2015). Two new targets, *PMP1* and *TPI1*, were successfully identified which improved 3-hydroxypropionic acid production. Genome-wide mutagenesis through DNA mutators or chemical mutation is another approach to generate libraries. Chou & Keasling constructed feedback-regulated devolution of phenotype by using a sensor to link the concentration of a metabolite to the mutation rate of *E. coli* (Chou & Keasling, 2013). This adaptive control system

activated the expression of mutator gene *mutD5* in the absence of the ligand, to generate a diverse population. Some of the mutations led to increased metabolite production, which in turn led to decreased mutation rate and reporter gene expression (Chou & Keasling, 2013). This technique resulted in a fivefold increase in tyrosine production and 17-fold increase in lycopene production (Chou & Keasling, 2013). In another study, Leavitt *et al.* designed a biosensor which coupled aromatic amino acid concentration to cell growth to drive anti-metabolite ALE in *S. cerevisiae* to improve muconic acid titers (Leavitt *et al.*, 2017). Ethyl methanesulfonate mutagenesis was performed and the biosensor enabled growth selective ALE to screen for mutants with improved aromatic amino acid production, thereby resulting in improved muconic acid yield.

Perspectives

Biosensors have proven to be powerful tools for metabolic engineering applications such as dynamic control and high-producer selection to generate robust microbial factories. Advances in genome engineering and synthetic biology facilitate our capability to build, engineer and apply biosensors. Currently, most yeast biosensors are transferred from bacterial biosensor systems or other eukaryotic systems. The operational and dynamic ranges may not be suitable for their application in yeast. Extensive studies are needed to optimize the features of such biosensors to improve their applicability. In addition, the types of metabolites that yeast biosensors can sense are still very limited. However, compared with prokaryotic biosensor systems, very few studies have focused on changing the ligand specificity of yeast biosensors. Therefore, more studies should be performed to engineer biosensors through both rational and randomized approaches.

Our ability to create genetic diversity has developed significantly. In vivo directed gene evolution technology like the orthogonal DNA polymerase-plasmid pair (OrthoRep) (Ravikumar *et al.*, 2018) or directed genome evolution technologies such as yeast oligo-mediated genome engineering (Dahl *et al.*, 2013) and RNAi-assisted genome evolution have been established in yeast (Si *et al.*, 2015). By coupling biosensors with continuous in vivo evolution or genome engineering, high-throughput analysis and screening can be achieved. Since biosensors have greatly accelerated the speed of high-throughput analysis and large data collection, with the advance of artificial intelligence, it is expected that new biosensors can be designed for more precise pathway optimization and cell factory development based on machine-learning algorithms with predictive power.

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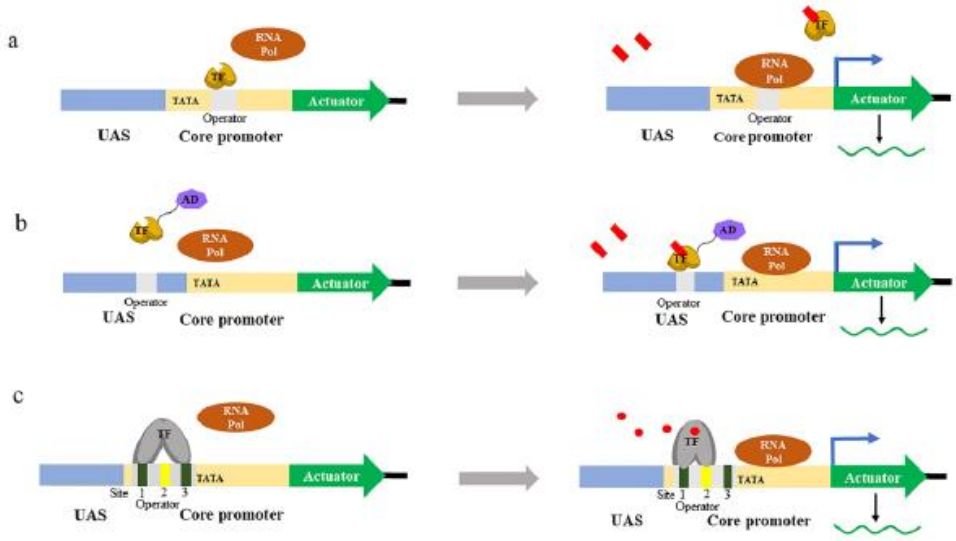

Fig.1. Introduction of heterologous transcription factors (TFs) for biosensor design in yeast. (a) A TF binds to the operator to block transcription in the absence of its ligand and dissociates from the operator to activate transcription in the presence of the ligand. (b) A hybrid transcriptional activator can be constructed when the TF is fused with an activation domain of a eukaryotic TF. Transcription is repressed in the absence of the ligand, and is activated when the hybrid transcriptional activator binds to the operator and recruits the transcription machinery in the presence of the ligand. (c) The presence of ligand triggers a conformational change of a prokaryotic transcription activator that leads to shifting of the binding site, promoting access of the transcription machinery and activating transcription.

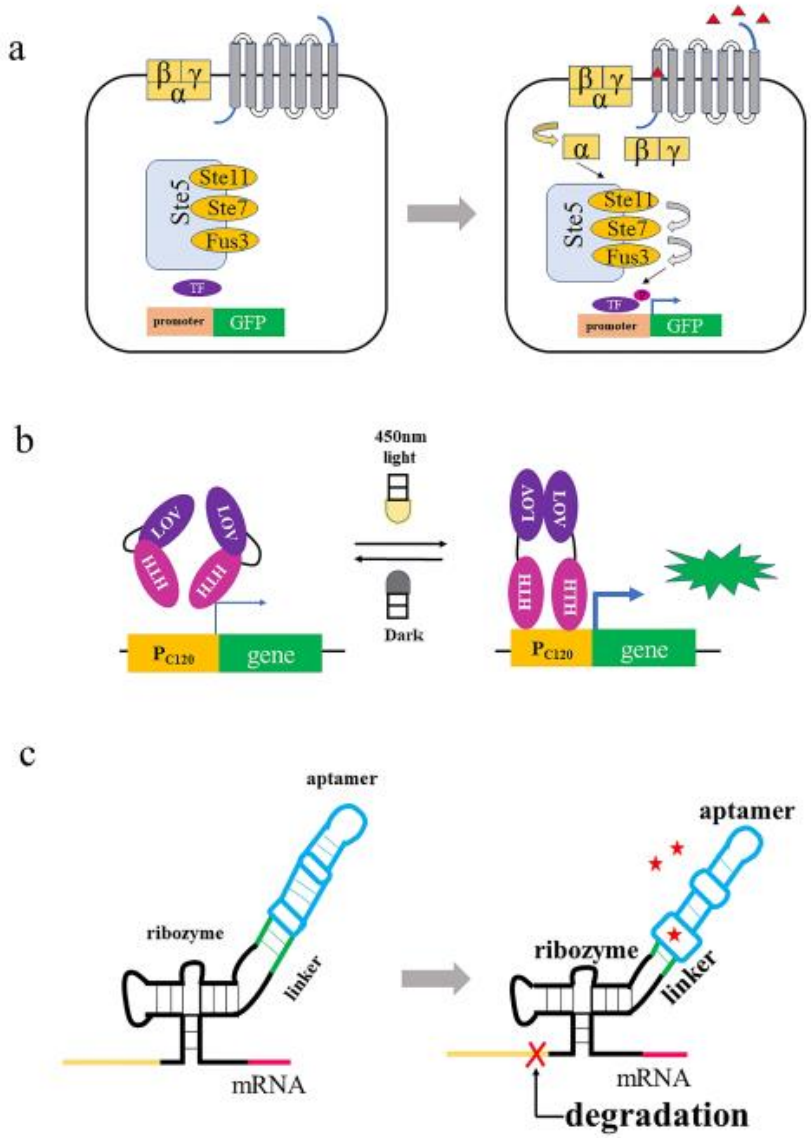

Fig.2. Representative biosensors that sense extracellular ligands or environmental changes, and RNA-based biosensors in yeast. (a) G-protein-coupled receptor (GPCR)-based sensors. The extracellular ligand binding binds to GPCRs on the cell surface, and induces a conformational rearrangement of the receptor, resulting in the dissociation of Gα from the heterodimeric β- and γ-subunits (Gβγ), which activates the downstream signaling pathway and finally activate the expression of reporter gene. (b) Optogenetic circuit design in yeast. The optogenetic system consists of a light-sensitive transcription factor and its corresponding C120 promoter (P_{C120}) which can sense 450 nm light to drive gene expression. (c) Ribozyme-based biosensors. The binding of the ligand causes the conformational change of the ribozyme to induce self-cleavage activity and destabilize the mRNA.

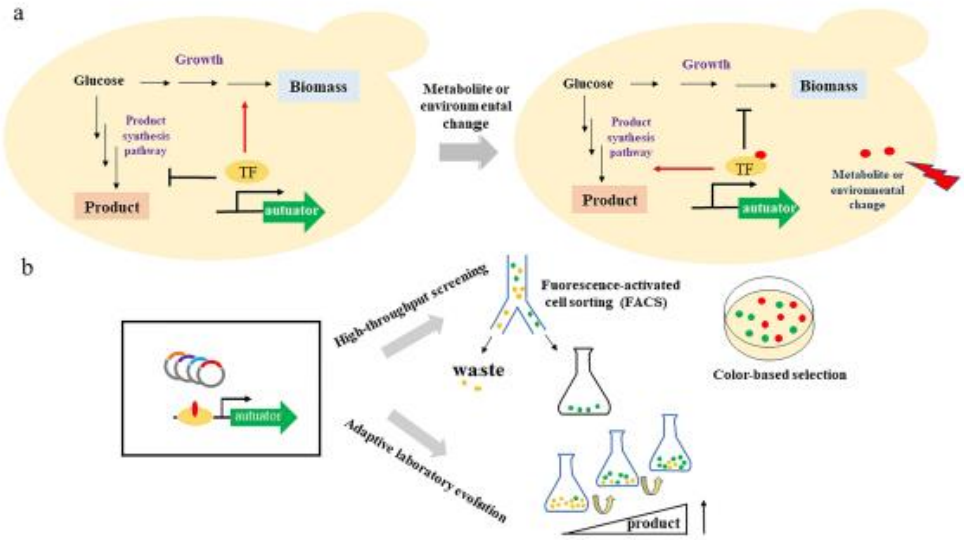

Fig.3. Metabolic engineering applications of biosensors in yeast. (a) Dynamic pathway regulation: A sensor-actuator system dynamically regulates product biosynthesis in yeast, balancing flux between cell growth and production of the target chemical. (b) High-producer selection: Coupling biosensors with the production of fluorescence or color enables high-throughput screening by fluorescence-activated cell sorting or color-based selection. In addition, coupling biosensors with cell growth enables high-producer selection by adaptive laboratory evolution.

Table 1. Examples of engineered biosensors in yeast

Type of sensor	Sensing element	Target molecules	Sources	Sensor design	Reference
TF	FadR	Fatty acid/acyl-CoA	*E. coli* and *Vibrio cholerae*	3x*fadO* immediately downstream of the *GAL1* TATA box promoter	(Wei *et al.*, 2014)
TF	FapR	Malonyl-CoA	*B.subtilis*	1x*fapO* immediately upstream the TATA box in *GPM1* promoter	(Li *et al.*, 2015)
TF	PhlF	DAPG	*Pseudomonas putida*	Transactivator PhlTA(*PhlF*-VP16) binds to *phlPr* promoter (*ADH1tr*-phlO-*CYC1pr*)	(Ikushima & Boeke, 2017)
TF	hAR	Androgen	*Human*	2xAREs placed in the truncated *CYC1* promoter	(Bovee *et al.*, 2007)
TF	CymR	P-Cumate	*P. putida*	Transactivator CymTA (NLS-*CymR*-VP16) binds to *CMV* promoter	(Ikushima & Boeke, 2017)
TF	CamR	Camphor	*P. putida*	Transactivator CamTA (*CamR*-VP16-NLS) binds to *CAM promoter (6xcamO -CYC1pr*)	(Ikushima & Zhao, 2015)
TF	XylR	Xylose	*Thellungiella halophila*, *Clostridium difficile*, and *Lactobacillus pentosus*	1x*xylO* immediately downstream the TATA box in $TEF1_{UEE}$-$GAL1_{core}$ promoter	(Teo & Chang, 2015)
TF	XylR	Xylose	*Caulobacter crescentus*	Repressor (NLS-XylR-Ssn6p) binds to hybrid promter ($P_{TEF\text{-}xylO2\text{-}1}$)	(Hector & Mertens, 2017)
	TetR-VP16			Transactivator tTA (tetR-VP16) binds to a	(Gari *et al.*, 1997)

TF	(tTA)	Doxycycline	*E. coli*	hybrid *tetO-CYC1* promoter	
TF	BenM	Cis,cis-muconic acid	*Acinetobacter* sp	1x*benO* immediately upstream the TATA box in *CYC1* promoter	(Skjoedt *et al.*, 2016)
TF	FdeR	Naringenin	*Herbaspirillu-m seropedicae*	1x*fdeO* immediately upstream the TATA box (TATA-1β) in *REV1* and *TDH3* promoter	(Skjoedt *et al.*, 2016)
TF	PcaQ	Protocatechuic acid (PCA)	*Sinorhizobium meliloti*	1x*pcaO* immediately upstream the TATA box in *CYC1* promoter	(Skjoedt *et al.*, 2016)
TF	ArgP	L-arginine	*E.coli*	1x*argO* immediately upstream the TATA box in *CYC1* promoter	(Skjoedt *et al.*, 2016)
TF	MdcR	Malonic acid	*Klebsiella pneumonia*	1x*mdcO* immediately upstream the TATA box (TATA-1β) in *CYC1* promoter	(Skjoedt *et al.*, 2016)
TF	MetJ-B42	S-adenosyl-methionine	*E.coli*	Transactivator (MetJ-B42) binds to a hybrid *metO-CYC1* promoter	(Umeyama *et al.*, 2013)
TF	GAL4DBD -Digoxin LBD-VP16	Digoxinand progesterone		Transactivator G-IG1-V (VP64 or VP16) binds to *CYC1* promoter	(Feng *et al.*, 2015)
TF	Ada-GAL4 AD	MeI-ethyl iodide	*E.coli*	Transactivator Gal4-N-Ada binds to a hybrid P_{CYC1} *promoter* ($P_{8x.CYC1}$)	(Moser *et al.*, 2013)
TF	IPP isomerase-GAL4 AD/IPP isomerase-GAL4	IPP	*S. cerevisiae*	synthetic TF consisting of Idi, GAL4 AD and DBD binds to upstream activation sequence (UAS) of P_{GAL10}	(Chou & Keasling, 2013)

	BD				
Ribozyme	Glucosamine 6-phosphate ribozyme	Glucosamine 6-phosphate	*B. subtilis*	The *glmS* ribozyme inserts the 3'-UTR of the *FCY1*	(Lee & Oh, 2015)
Ribozyme	A synthetic neomycin aptamer	Neomycin	*Schistosoma mansoni*	The hammer headribozyme inserts the 3'-UTR of the *GAL4*	(Klauser *et al.*, 2015)
Enzyme-coupled	α-Ketoglutarate dehydrogenase	Free CoA-SH	*Solanum lycopersicum*	The activity of AATase was measured by the generation of NADH through α-ketoglutarate dehydrogenas (α-KGDH)	(Lin *et al.*, 2016)
Enzyme-coupled	DOPA dioxygenase	L-3,4-dihydroxy phenylalanine	*Mirabilis jalapa*	DOPA dioxygenase converted L-DOPA to yellow pigment	(DeLoache & Russ, 2015)
GPCR	OR1G1 and GPR40	Medium-chain fatty acid	*S. cerevisiae*	Olfactory receptor OR1G1 and the free fatty acid receptor GPR40	(Mukherjee *et al.*, 2015)
GPCR	5-HT4	Serotonin	*S. cerevisiae*	Olfactory receptor OR1G1	(Ehrenworth *et al.*, 2017)