Review

Engineering of Synthetic Transcriptional Switches in Yeast

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Abstract: Genetic switches can be utilized for many purposes in synthetic biology including the assembly of complex genetic circuits to achieve sophisticated cellular systems and the construction of biosensors for real-time monitoring of intracellular metabolite concentrations. Although genetic switches have mainly been developed in prokaryotes to date, eukaryotic genetic switches are increasingly being reported as both rational and irrational engineering technologies mature. In this review, we describe genetic switches in yeast based on synthetic transcription factors and/or synthetic promoters. We also discuss directed evolution technologies for the rapid and robust construction of yeast genetic switches.

Keywords: genetic switch; yeast; synthetic transcription factor; synthetic promoter; directed evolution

1. Introduction

In the field of synthetic biology, it is necessary to develop genetic elements such as promoters and terminators to fulfill the increasing demand for precise control of complex gene expression, which is required for the construction of sophisticated biological systems (1). In particular, genetic switches are required to regulate gene expression in response to intracellular and extracellular stimuli (e.g., metabolites and inducers) and thereby modulate cellular phenotypes via their output (2). To date, genetic switches have been utilized to build inducible expression systems (3, 4, 5, 6), genetic circuits (7, 8, 9, 10, 11), and metabolite sensors (12, 13, 14, 15, 16). Unfortunately, however, most genetic switches cannot be used directly for synthetic biology applications because of their inappropriate switching properties. For example, such promoters often exhibit detectable activity in an OFF-state (leaky expression), which hampers the expression of toxic proteins (17). Besides, if genetic switches are to be used as elements of a synthetic bioengineering toolbox, they must be designed to respond specifically to the desired target chemical with minimal cross reactivity, no leakiness, and sufficient output when activated; otherwise, the construction of complex, higher-order, genetic circuits, especially with layered logic gates, will fail (18).

Endogenous systems such as the Gal4 transcriptional switch (3, 4, 5) and G-protein coupled receptor systems (19) can be used as genetic switches in yeast. Alternatively, genetic switches can be artificially created using heterologous, ligand responsive, DNA binding proteins, exemplified by bacterial transcription factors (TFs), which can be used to control promoter activity. Tet-ON and Tet-OFF systems that regulate gene expression in response to a small molecule, doxycycline (Dox), were first described as synthetic genetic switches for mammalian systems (20) and subsequently used in various fungi (21, 22, 23). Yeast genetic switches can also be created at the translational level using aptamers, riboswitches, and ribozymes; this was recently reviewed by Ge and Marchisio (24).

In the present review, we describe how synthetic transcription-level genetic switches have been created in yeast. Synthetic genetic switches in yeast are categorized according
to two types of regulation: a transcription activation/deactivation mode with synthetic transcription activators (sTAs) and a transcription repression/derepression mode with bare transcription repressors (Fig. 1). We also describe strategies to improve the switching performance of each genetic switch with different modes of action. Finally, we present the evolutionary techniques used to improve or create the functional genetic switches in yeast, especially in *Saccharomyces cerevisiae*.

**Figure 1. Construction of yeast genetic switches using synthetic transcriptional activators or bare transcription factors.** Inducer-ON and inducer-OFF types of genetic switches can be constructed using sTA (A and B) and intact bacterial repressors (C, D and E), respectively. C. Transcription activation based on bacterial transcription activators. Ligand binding to transcription activators induces conformational changes to promoters and the recruitment of RNA polymerase.
Abbreviations: sTA, synthetic transcription activator; TF, transcription factor; TFrev, reverse transcription factor; eTA, eukaryotic transcription activator; RNAP, RNA polymerase; TATA, TATA Box; synP, synthetic promoter; GOI, gene of interest.

2. Synthetic transcriptional switches with different modes of regulation in yeast

2.1. Transcription Activation/Deactivation Mode

Although transcription from a yeast promoter requires the recruitment of multiple endogenous TFs to the promoter, binding of a single protein fused with eukaryotic transcription activators (eTAs) is sufficient to artificially stimulate the recruitment of yeast TFs. Therefore, synthetic transcriptional switches can be created in yeast by fusing eTAs with ligand-responsive DNA binding proteins such as bacterial TFs (bTFs; also known as allosterically regulated TFs). With the appropriate design parameters, ligand-dependent binding of bTFs to their operator DNA sequences fused upstream of the yeast core promoter [i.e., a yeast promoter lacking an upstream activation sequence (UAS)] can be translated to the output gene expression (20, 23, 25, 26, 27) (Fig. 1A and 1B).

2.1.1. sTAs Based on Transcription Repressors

The Tet-OFF system (22, 23) is a famous and proven technology that uses a synthetic transcription activator, i.e., a tetracycline-controlled transactivator (tTA), to activate target gene expression. For example, a system incorporating a bacterial TetR repressor facilitates the control of strongly inducible expression in response to Dox, a more stable and less expensive analog of tetracycline (28). The tetracycline-responsive system has been used extensively as a synthetic genetic switch in gene circuits (29, 30, 31, 32); in particular, it has been used to explore the function of genes that confer toxic phenotypes because the switches provide regulated expression of the candidate genes (33, 34). Recently, variants of the tetO sequence that binds to TetR with different affinities were developed by Fields’ group as a toolkit for generating varying levels of gene expression (35). Using this toolkit, gene expression for carotenoid biosynthesis was tested and production of lycopene at high levels was successfully demonstrated.

Bacteria harbor various TetR homologues (36); the corresponding operator sequences and ligand molecules have been identified for some of these repressor proteins. A series of distant orthogonal genetic switches is a prerequisite to the independent and arbitrary regulation of multiple genes or pathways via different ligands. Boeke’s group recently engineered distant homologues of the Escherichia coli TetR repressor from several species; thus, they developed new genetic switches that tightly control gene expression in S. cerevisiae (26, 27). In addition, Ikushima et al. have developed a genetic OFF switch that is tightly controlled in S. cerevisiae by camphor, an inexpensive small molecule (26). The transcriptional repressor CamR, a TetR homologue from Pseudomonas putida, binds to its operator sequence (camO) in the absence of camphor; however, it dissociates from the operator in response to the binding of the camphor ligand, which leads to the induction of the cytochrome P-450cam hydroxylase operon (camDCAB) (37, 38). In this case, an sTA was created by fusing three tandem repeats of the eTA (VP16) and a nuclear localization sequence (NLS) to the CamR transcriptional repressor. A corresponding synthetic promoter was also created by embedding six repeats of camO operator sequences between the ADH1 terminator (to avoid leaky crosstalk from upstream transcription) and the CYC1 minimal promoter (which lacks the UAS). The resulting system (the Camphor-OFF switch) facilitates camphor-dependent regulation of downstream gene expression via an “inducer-OFF”-type sTA (Fig. 1A). In this system, the sTA promotes transcription in the absence of camphor because ligand-free CamR binds to the camO operator elements. In contrast, the transcription remains OFF in the presence of camphor because camphor-bound CamR dissociates from the camO elements. In subsequent research conducted by the same group (27), a series of “inducer-OFF”-type genetic switches that function in S. cerevisiae were created using different TetR homologue transcription repressors and their cognate opera-
tor DNAs and ligands: the DAPG-OFF switch [PhlF/phlO from *Pseudomonas* and 2,4-diace
tetylphloroglucinol (DAPG) (27)] and the Cumate-OFF switch (CymR/cymO from *P. putida*
and *p*-cumate). Recently, the same design was applied to the construction of a malonyl-
CoA sensor based on the transcription repressor FapR from *Bacillus subtilis* both in *S. cere
visiae* (39) and the nonconventional yeast *Komagataella phaffii* (40). In addition, the design
was used to construct a xylose sensor based on the transcription repressor XylR from *Staphylo
coccus xylosus* (39). Upon binding SAM, the repressor protein MetJ from *E. coli* binds to
its operator DNA, metO. Thus, a MetJ-based biosensor for SAM was created by fusing
MetJ with eTA (B42), and by fusing *metO* with the CYC1 promoter upstream of the TATA
box to create a synthetic promoter, namely *metO*-pCYC1.

Several eTAs, including VP16, B42, and Gal4, have been chosen for sTAs. Although
many synthetic transcription activators have been constructed in yeast, as described
above, eukaryotic activation motifs were not systematically compared until recently. In
2020, Qiu et al. compared the different transcription activation motifs [i.e., Gal4, Med2,
VP16, VP64-p65-Rta (VPR), and Med2-Gal4] fused to the FapR repressor in terms of their
activation capacities; they found that Med2 outperformed the other motifs in *S. cere
visiae* (39).

### 2.1.2. sTAs Based on Ligand-induced DNA Binders

Mutant sTAs can be used to create genetic switches with reversed switching behavior
(Fig. 1B). The first report of a reversed sTA was a reversed tTA (rtTA) based on reversed
TetR that binds to *tetO* in the presence of Dox. The discovery and evolutionary engineer-
ing of rtTA have been reviewed previously (28). The rtTA was first found in the directed
evolution of *E. coli* (20) and then found in yeast (42). Subsequently, improved Dox-sensi
tivity was achieved using directed evolution in yeast (42) and then using viral evolution
(43). In addition, Roney et al. serendipitously discovered a mutation in rtTA that signifi-
cantly reduces leaky activation of rtTA (44). Recently, using a novel directed evolution
platform described later (45) (section 3.4), we found a reverse PhlF mutant (rPhlF: PhlF
with K86T, Q117R, and E143K) that binds to *phlO* in the presence of DAPG. We also found
that the Q117R mutation in PhlF causes the reversed phenotype. A switch utilizing a syn-
thetic transcription activator that was developed using this mutant PhlF showed >100-fold
induction upon DAPG addition, which is comparable to the effect of a well-optimized Tet-
ON system.

In addition to mutant repressors, bacterial transcriptional activators are known to be
ligand-triggered DNA binders. Moser et al. first described a synthetic biosensor that could
sense methylating compounds, such as methylnitronitrosoguanidine and methyl me-
thanesulfonate, in yeast (46). In this system, the N-terminal region of the Ada from *E. col*
i was fused with the Gal4 transcription activation domain. Upon addition of methylating
compounds, Ada protein is methylated to bind to the cognate operator sequence, which
facilitates the transcriptional activation of the synthetic promoter. Later, Castano-Cerezo
et al. engineered a 4-hydroxybenzoate responsive transcription activator, HbaR, from *Rho
dopseudomonas palustris* into an sTA by fusing the protein with a transcription activator,
B12, and DNA binding protein, LexA (47). Wei et al. reported the fusion of a xylose re-
sponsive transcription activator, XylR, from *E. coli* with a eukaryotic transcription activa-
tion motif, VPR, or heat shock factor 1 to create a xylose sensor both in *S. cerevisiae* and
the oleic yeast *Yarrowia lipolytica* (48). In a recent example, we described the first yeast sTA
based on a bacterial quorum-sensor protein, namely LuxR (45). Specifically, the TetR-fam-
ily transcription activator LuxR was fused to three copies of VP16 (VP48) to activate a
synthetic promoter in yeast composed of the *GAL1* core promoter fused with 1–15 copies
of the LuxR binding sequence (*luxO*). This system exhibited >100-fold induction upon ad-
dition of a quorum signal, i.e., 3-oxo-hexanoyl homoserine lactone (HSL). Notably, using
directed evolution of LuxR to improve its sensitivity toward HSL in yeast enabled the
identification of a novel sensitizing mutation that abolishes the native function of LuxR as
a transcriptional activator in bacteria. This mutation would not be found during evolutionary engineering in a prokaryotic system where the transcriptional activation of LuxR is essential for the function of a genetic switch.

2.1.3 sTAs Based on Ligand-responsive Nuclear Localizers

sTAs can also be created with eukaryotic receptor proteins (49, 50, 51, 52, 53). In one example, Chockalingam et al. demonstrated an sTA based on human estrogen receptor fused with both a DNA binding protein and transcription activation motif (49). In the absence of its native ligand, β-estrogen (including β-estradiol), the host Hsp90 chaperon complex binds to the sTA and prevents it from being transported to the nucleus (i.e., it remains in the cytosol). Upon ligand binding, the sTA is released from Hsp90 and transported into the nucleus to bind to the target DNA sequence; this results in the activation of target gene transcription. These authors mutated the ligand binding domain of the receptor to completely alter ligand specificity. Four rounds of saturation mutagenesis to the ligand binding domain and one round of whole-gene mutagenesis followed by genetic selection and screening enabled the authors to identify the mutant that was specifically responsive to the non-native ligand 4,4’-dihydroxybenzil (DHB). More recently, Mormino et al. developed a sTA based on the fusion of an acetic acid responsive transcription factor from *S. cerevisiae*, Haa1, and a DNA binding protein, BM3R1, from *Bacillus megaterium* (54). In this system, Haa1p is relocated to the nucleus following the binding of acetic acid, which causes binding to the BM3R1 binding sequence fused upstream of the yeast promoter. With this acetate-responsive genetic switch, it is possible to monitor the acetic acid concentration in yeast within a linear range from 10 to 60 mM.

2.1.4. Transcription Activation Without a Eukaryotic Activation Motif

Skjoedt et al. built biosensors based on bacterial LysR-type transcriptional regulators (Fig. 1C) (55). For systematic prototyping, they used BenM from *Acinetobacter* sp. ADP1, which responds to *cis*, *cis*-muconic acid (CCM), and its cognate operator DNA, benO. In the native system, BenM homotetramer binds to its cognate suboperators [sites 1 and 3 (S1 and S3)] even in the absence of CCM. When CCM binds to BenM, CCM-bound BenM homotetramer binds to different suboperators [sites 1 and 2 (S1 and S2)], which alters the DNA conformation and thereby enhances RNA polymerase (RNAP) binding to the bacterial promoter. Three synthetic promoters were created by fusing the yeast CYC1 promoter with a single benO at different positions; one of the fusions with benO at position –106 (upstream of the TATA box) resulted in a 20-fold increase in promoter activation upon constitutive expression of BenM from the TEF1 promoter. Surprisingly, this activation did not require the presence of a eukaryotic activation motif. With an evolutionarily optimized BenM mutant (H110R, F211V, and Y286N), cells exhibit a 10-fold increase in induction following the addition of 1.4-mM CCM. This synthetic promoter configuration has been used to construct three different biosensors by using the operators of FdeR (*fdeO*), ArgP (*argO*), MdcR (*mdcO*), and PcaQ (*pcaO*) instead of benO, which respond to naringenin, L-arginine, and malonic acid, protocatechuic acid, respectively (55, 56). In subsequent research by the same group, using fluorescent-activated cell sorting (FACS)-aided directed evolution of BenM enabled the identification of a BenM mutants with reversed switching phenotype (CCM-induced deactivation; CCM-OFF), improved induction-fold and operational range, and altered ligand specificity (16).

2.2. Transcription Repression/Derepression Mode

When an intact bacterial transcription repressor binding sequence is fused to a position upstream of the TATA-box in a yeast promoter, transcription is blocked by inhibition of RNAP complex binding. The earliest example of this is a Dox-inducible system based on TetR and tetO, namely Tet-ON, in which intact TetR binds to tetO and hinders the binding of RNAP to the promoter (transcription repression). Following the binding of Dox
to TetR, the latter dissociates from the operator, resulting in the derepression of the synthetic promoter (Fig. 1D). With the aim of generating a diversity of promoters to enable fine-tuning of expression, the Ellis laboratory created a similar inverter device by placing the TetR regulatory machinery under the control of the highly expressed constitutive PFY1 promoter, which was identified using bioinformatics (57). Any transcription repressor and its binding target sequence from bacteria can be assembled into this type of genetic switch. Ikushima and Boeke demonstrated repressor-based sensing using the native PhlF repressor without the VP16 transcriptional activation domain (the DAPG-ON switch) (27) (Fig. 1E; the “inducer-ON”-type transcriptional repressor). One or two phlO elements were embedded downstream of the constitutive ADH1 promoter and then the NLS-attached PhlF was constitutively expressed in yeast. In the absence of DAPG, ligand-free PhlF binds to the phlO elements (without DAPG), which represses reporter transcription; in the presence of DAPG, DAPG-bound PhlF dissociates from the phlO elements, which permits the initiation of reporter transcription. In principle, it should be possible to create an “inducer-OFF”-type transcriptional repressor using, for example, a mutant transcriptional repressor (rtTA and rPhlF) exhibiting opposing binding behavior (as described in section 2.1.2).

D’Ambrosio et al. built a vanillin-responsive yeast genetic switch based on a vanillin-responsive transcriptional regulator, vanR from Caulobacter crescentus using systematic promoter engineering and FACS-assisted directed evolution; this switch enabled up to 3-fold induction in the presence of 4-mM vanillin (15). In another study from the same group, Ambri et al. screened over 300 promoter designs to identify the optimal positions in synthetic promoters for three different bTF-binding sites (benO, vanO, and pcaO for BenM, VanR, and PcaQ, respectively) at single-nucleotide resolution in S. cerevisiae; the optimal insertion position was highly dependent on the type of bTF (56). Nevertheless, it has been possible to create such synthetic systems using the various inducer responsive transcription factor, XylR (58, 59, 60), FdeR (61), FadR (62), and FapR (63), which respond to xylose, naringenin, fatty acids, and malonyl-coA, respectively. For XylR, the choice of bTF–operator combinations substantially affected the switching performance; following xylose addition, the maximum fold induction was obtained with a XylR/operator pair from B. subtilis (59). Furthermore, the same design has already be applied to different yeasts, e.g., fission yeast (64) and the methylotrophic yeast K. phaffii (65). In the latter instance, Cao et al. inserted the LacI binding sequence (lacO) downstream of the GAPDH promoter, which resulted in a 6-fold induction of gene expression when isopropyl-β-D-thiogalactopyranoside (IPTG) was added (65). Recently, Gita et al. reported LacI/lacO-based regulation in combination with an artificial transcription activator based on a plant-derived TF; this system resulted in up to a 63-fold induction upon IPTG addition in K. phaffii (66).

To reduce the leakiness of regulated promoters, chromatin remodeling modules, such as Ssn6, can be fused to TFs. The effectiveness of this method was verified by constructing genetic switches with XylR-Ssn6 (60) and TetR-Ssn6 (22) fusion. Recently, Chen et al. performed rule-based optimization of this type of genetic switch (10). First, they investigated the minimal synthetic promoter sequence that had strong activity independent of nutrient conditions; the promoter with a 20-bp poly(T) sequence 4 bp upstream of the TATA box met these criteria. They also found that blocking transcription readthrough by placing appropriate yeast terminators and ribozymes upstream of the regulated promoters could minimize the leakiness of the repressible genetic switches. Besides, the tightest repression by bTF was found to be achieved by separating two operators more than 20 bp. Using these design strategies, they developed three strongly repressible switches with >100-fold induction and low basal output. Furthermore, they applied the resultant genetic switches with a wide-dynamic range to automatically assemble yeast genetic circuits.

3. Directed Evolution of Genetic Switches in Yeast

As described above, any ligand-responsive DNA binding protein and its binding sequence can be assembled with yeast promoters to build synthetic gene regulation systems
in yeast; however, the prototype switches often perform poorly. Researchers must identify the appropriate expression level of the sTAs or repressors by changing promoters and terminators (44). The optimal operator number and positions should be screened in a single base resolution (15, 25). The choice of yeast promoter sequence used to construct synthetic promoters via operator fusion inside/outside the promoters can substantially affect their performance (56, 59, 67). The tuning process of eukaryotic genetic switches is far more difficult than the process required for prokaryotic cells because the behaviors of eukaryotic promoters are easily affected in an unpredictable manner by changes to surrounding sequences (68, 69). For example, the inducer sensitivity of a genetic switch may be altered by several orders of magnitude when it is transferred into different contexts (70); thus, to achieve desired performance levels, eukaryotic switches require extensive effort over many years to fine-tune expression and/or protein-engineer sTAs as well as to rearrange the sequence of the synthetic promoter, which was the case for the commonly used Tet-ON/Tet-OFF systems (23, 25, 28).

Directed evolution is one of the tractable strategies used to develop useful transcriptional controllers in yeast. Genetic switches with desired performances are designed to be selected from the genetic switch library with randomized components. This strategy has been widely used in prokaryotic systems, especially in E. coli, resulting in a large genetic switch toolbox. In contrast, only a few examples of this evolutionary engineering strategy have been reported in yeast, despite it being routinely employed in E. coli (1, 71, 72, 73, 74, 75, 76, 77). In the following subsections, we summarize the recent successes in the evolutionary engineering of genetic switches in yeast as well as the methodologies used in each example.

3.1. Fluorescence-based Screening

Most genetic switches have been evaluated by placing genes for fluorescent protein as an output. Fluorescence measurements are then taken using multiwell-based plate readers or flow cytometry. Ellis et al. developed a tetracycline-responsive genetic switch in yeast by fusing the GAL1 promoter with two distinct tetO sequences where the TetR protein binds to repress transcription from the GAL1 promoter (78). They found switch variants with the optimal characteristics for construction of different genetic circuits (a feed-forward loop and timer) by performing green fluorescent protein (GFP)-based screening of synthetic promoter variants for which the sequence around the two tetOs were randomly mutated. Urlinger et al. performed GFP-based screening of approximately 1,000 clones on plates with and without Dox with the aim of identifying tTA mutants with a reversed phenotype (42). Furthermore, they performed a second round of mutagenesis and screening to identify rtTA mutants with improved sensitivity. As discussed in a previous review article (79), GFP-based screening is particularly important when the cells show clonal populations. FACS-based screening, rather than agar-plate or plate reader-based screening, can be essential.

3.2. ON/OFF Selections Using FACS

FACS enables the enrichment of cells with desired output levels at given conditions of from $10^3$ to $10^5$ variants per second (80, 81, 82, 83). This allows for high-throughput selection of vast libraries of genetic switches. With a FACS-based selection strategy, directed evolution of the responsivity of bacterial activators that are responsive to muconate (BenM) (16, 55) and vanillin (VanR) (15) were performed to obtain mutants with the specifications described above (sections 2.1.4 and 2.2). Because FACS-based selection can be performed with tunable selection thresholds by changing gating conditions, it is independent of the specifications of prototype sensors. However, selection efficiency is highly dependent on both selection and gating conditions. Thus, sorting experiments must be repeated until the correct selection/gating condition is achieved given the lack of a priori knowledge related to the necessary selection conditions for each system.
3.3. ON/OFF Selections Using Genetic Selections

Positive and negative selection can be used to enrich genetic switches with defined outputs under ON and OFF conditions. Positive selection markers include auxotrophic markers [e.g., His3/3-aminotriazole (3-AT)] (84), antibiotics markers (e.g., Ble/Zeocin KanMX/G418) (85), and counter selectable markers (86) such as Ura3/5-fluoroorotic acid (5-FOA) (84) and herpes simplex virus thymidine kinase (hsvTK)/5-fluorodeoxyuridine (5FdU) (87). Directed evolution of genetic switches using genetic selection was first demonstrated by Chockalingam et al., who converted 17β-estradiol responsive human receptor into a synthetic nonsteroidal compound, namely DHB responsive receptor (50). The synthetic transcription activator based on the mutant hormone receptor could be grown on a medium lacking histidine in the presence of DHB. Later, Klauser et al. demonstrated the use of both ON/OFF selections to enrich functional translational switches (84). In their system, the product of HIS3, imidazole glycerol phosphate dehydratase, was gradually inhibited dependent on the concentration of its inhibitor, 3-AT. Titration of 3-AT concentrations enabled the enrichment of cells with higher outputs under the ON-state (ON selection). OFF selection was based on the URA3, coding orotidine 5-phosphate decarboxylase; in the presence of 5-FOA, the cells expressing more URA3 converted more 5-FOA into the toxic compound 5-fluorouracil, which enabled the enrichment of cells without any leaky URA3 expression in the absence of the ligand.

3.4. “Screening of Selection” Strategy

Despite the successes described above, the evolutionary strategy has not been widely applied to genetic switches in yeast. As previously described (79), stringent selection can be performed when genetic switches to be selected outcompete other nonfunctional (always-ON and always-OFF) variants; however, it is more difficult to select for mutants with a distinct ON-state output from the majority of variants with slightly weaker output or vice versa. In most cases, even low expression levels of ON and OFF selection markers are already sufficient to allow cell growth and to cause cell death, respectively, which results in low selection efficiency. Moreover, genetic selection fails to discriminate desired genetic switches from others when their output levels under selection are too far off the given selection threshold (84, 88). Thus, it is important to identify the appropriate selection conditions that selectively enrich rare mutants with slightly improved performance. To meet this challenge, researchers must reconstruct the selector systems in almost every directed evolution cycle. However, even though the selection pressure of the His3/3-AT system is tunable by altering 3-AT concentration, reconstruction of the selection system is required to further optimize the selection pressure in some cases (84, 88). FACS-based selection is more capable of fine-tuning selection pressure, but the lack of a priori knowledge related to appropriate gating conditions for each system is a major obstacle to improving the application of this strategy.

One possible solution to this problem would involve performing multiple selections for different selection pressures in parallel and choosing the most promising selection pools from which the improved mutants would be screened (i.e., “screening of selection conditions”). We recently described an evolutionary platform for yeast genetic switches that could meet this demand we used a trifunctional fusion protein, consisting of hsvTK, Zeocin-resistance protein (Ble), and GFP (45). This fusion protein, hsvTK-Ble-GFP facilitates seamless ON/OFF selections with different selection pressures and only liquid handling in parallel using multiwell plates. Among the liquid-based ON selections, the combination of Ble and Zeocin is intrinsically suitable for genetic switches because the Ble dimer inactivates Zeocin by sequestering it from DNA at a 1:1 molar ratio; thus, the survival rate in the presence of Zeocin resistance of a cell should directly reflect the expression level of Ble. For OFF selection, we found that using hsvTK and the synthetic nucleoside 5FdU enables a progressive decrease in cell viability in proportion to the genetic switch output. The ON/OFF-selected cell populations can be characterized seamlessly us-
ing flow cytometry, by which promising populations can be identified, from which improved genetic switch mutants can be screened. As a demonstration of this platform, we were able to isolate Dox-ON switches with improved fold induction ability by performing 14 selection conditions in parallel from which two promising conditions were subjected to further screening. Furthermore, we used the platform to develop a series of novel genetic switches with different specifications, namely DAPG-ON and HSL-ON switches (as described in section 2.1.1).

4. Conclusions

As described in this review, a number of bacterial TFs can easily be assembled into yeast genetic switches with or without the use of an evolutionary strategy. This method is now becoming the gold-standard for the engineering of genetic switches in yeast; however, rational engineering for the functional tuning of each component, which is crucial for maximizing genetic switch performance, is not feasible to date. Because the evolutionary platform for genetic switches in yeast has recently been demonstrated (15, 45, 55), the number of such synthetic switches has rapidly increased, which highlights the potential of prokaryotic TFs as an untapped resource for yeast genetic switches. Besides, Umeno et al. argued that TFs are intrinsically evolvable; therefore, mutants that are responsive to non-native compounds can be reached within a few directed evolution cycles (89). Indeed, dozens of new or evolved genetic switches (biosensors) based on bacterial TFs have been reported within the last 2 years (1, 90, 91, 92, 93, 94, 95, 96, 97, 98). Library creation guided by machine-learning technology combined with deep mutagenesis and extensive sequencing technology will further accelerate the discovery of novel TF mutants (98). TFs created in this manner will be applied to yeast and then further evolved in yeast, possibly with the aid of in vivo autonomous mutagenesis techniques (99, 100, 101) as well as automated continuous evolution technologies (102).

The genetic switches of yeast that can sense intracellular metabolites, i.e., metabolite sensors, are beginning to be used in high-throughput screening of higher metabolite-producing cells (103, 104) and in the metabolite-responsive dynamic control of enzyme expression in yeast (105) (which was also recently reviewed (106, 107)). These applications require that genetic switches can be optimized (evolved) depending on their purpose. For example, continuous evolution of cis,cis-muconic acid production in yeast uses the evolved sensory protein BenM (105). Besides, because yeast genetic switches and their assembly (genetic circuits) are based on bacterial TFs optimized in yeast, they are readily transferable into mammalian systems (53, 108). Expansion of the well-optimized (evolved) yeast genetic switches reviewed herein will result in the accelerated development of more complex mammalian systems. Taken together, directed evolution platforms for genetic switches in yeast can be one of the key technologies that push forward eukaryotic synthetic biology.

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