Title

A combinatorial approach to synthetic transcription factor-promoter combinations for yeast strain engineering

Journal

Yeast, 35(3)

ISSN

0749-503X

Authors

Dossani, Zain Y

Apel, Amanda Reider

Szmidt-Middleton, Heather

et al.

Publication Date

2018-03-01

DOI

10.1002/yea.3292

Peer reviewed

A combinatorial approach to Synthetic Transcription Factor-Promoter combinations for yeast strain engineering

Zain Y Dossani[1,2#], Amanda Reider Apel[1,2#], Heather S. Middleton[1,2], Nathan J. Hillson[1,2,3,4], Samuel Deutch[2,3,4], Jay D. Keasling[1,2,5,6], Aindrila Mukhopadhyay[1,2 *]

[1]DOE Joint Bioenergy Institute, Emeryville, California, USA

[2]Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, California, USA

[3]DOE Joint Genome Institute, Walnut Creek, California, USA

[4]DOE Agile BioFoundry, Emeryville, California, USA

[5]Department of Chemical & Biomolecular Engineering and Department of Bioengineering, University of California, Berkeley, CA, USA

[6]Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Building 220 Kemitorvet, 2800Kgs Lyngby Denmark

# Equal contribution

* Corresponding author: Aindrila Mukhopadhyay, amukhopadhyay@lbl.gov

Running Title: Synthetic Transcription factor-Promoter combinations for yeast

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/yea.3292

Abstract

Despite the need for inducible promoters in strain development efforts, the majority of engineering in *Saccharomyces cerevisiae* continues to rely on a few constitutively active or inducible promoters. Building on advances that use the modular nature of both transcription factors and promoter regions, we have built a library of hybrid promoters that are regulated by a synthetic transcription factor. The hybrid promoters consist of native *S. cerevisiae* promoters, in which the operator regions have been replaced with sequences that are recognized by the bacterial LexA DNA binding protein. Correspondingly, the synthetic transcription factor (TF) consists of the DNA binding domain (DBD) of the LexA protein, fused with the human estrogen binding domain and the viral activator domain, VP16. The resulting system with a bacterial DBD avoids the transcription of native *S. cerevisiae* genes, and the hybrid promoters can be induced using estradiol, a compound with no detectable impact on *S. cerevisiae* physiology. Using combinations of one, two, or three operator sequence repeats and a set of native *S. cerevisiae* promoters, we obtained a series of hybrid promoters that can be induced to different levels, using the same synthetic TF and a given estradiol. This set of promoters, in combination with our synthetic TF, has the potential to regulate numerous genes or pathways simultaneously, to multiple desired levels, in a single strain.

Introduction

The yeast *Saccharomyces cerevisiae* is one of the most well-understood and widely used organisms in biological research as well as in bio-production processes. In addition to natively produced ethanol, *S. cerevisiae* has been engineered to produce a wide variety of products including pharmaceuticals, fuels, and industrial chemicals [Peralta-Yahya, Zhang, del Cardayre, and Keasling 2012]. For optimizing the production of bulk chemicals, multiple traits, in addition to an optimal biosynthetic pathway must be developed [Lechner, Brunk, and Keasling 2016]. These necessitate the use of multigenic pathways and genes that modulate other phenotypes, such as for tolerance to the final product [Mukhopadhyay 2015], pretreatment reagents [Frederix et al. 2014] and optimal carbon uptake [Reider Apel, Ouellet, Szmidt-Middleton, Keasling, and Mukhopadhyay 2016]. Yet, the number of promoters used for genetic engineering in *S. cerevisiae* have remained limited to a few dozen native promoters; either constitutive promoters or galactose-inducible promoters that are the staple of the yeast genetic engineer's toolbox [Alper, Fischer, Nevoigt, and Stephanopoulos 2005; Lee, DeLoache, Cervantes, and Dueber 2015; Reider Apel et al. 2017]. In cases where promoter inducibility is desired, galactose induction is particularly problematic because of the limitations it places on the types of carbon sources that can be used for cultivation.

Both promoter sequences and transcription factors (TF) are modular in nature. The first effort to use this aspect of regulatory regions and DNA binding proteins was a synthetic TF for *S. cerevisiae* reported in 1993 [Louvion, Havaux-Copf, and Picard 1993] that used the Gal4 protein as the scaffold. By simply replacing the ligand binding domain of Gal4 with a human estrogen binding domain (hEF) and using the VP16 viral activators protein that recruits the RNA Pol II complex, the authors could obtain gratuitous regulation of galactose responsive genes in response to estradiol. Though promising, this system was not used in any reported metabolic engineering efforts, possibly due to the fact that the TF binds to native Gal responsive promoters, leading to crosstalk and unwanted changes in metabolism. More recently, McIssac and coauthors developed a similar system, where they replaced the Gal4 DNA binding domain (DBD) with zinc-finger DBDs [McIsaac, Oakes, Xin Wang, Dummit, Botstein, and Noyes 2013]. Further, using a variable number of operator sequences in the corresponding promoters, they obtained superior dynamic range for the synthetic TF and promoter combinations. Yet, the system suffers from low controllability at low to mid-range expression levels, due to high basal

activity of the zinc fingers [McIsaac, Gibney, Chandran, Benjamin, and Botstein 2014]. Similarly, Ottoz et al have developed a variation that uses the DBD from the bacterial TF, LexA. The authors tested several variations of the LexA-based TF where they varied the activating domain to obtain a range of highly regulated TF- promoter combinations, adjusting the output by increasing the number of LexA-binding sites, without changing basal promoter activity [Ottoz, Rudolf, and Stelling 2014]. Another recent study, achieved a similar range of expression levels without the need for externally added inducer compounds [Rantasalo, Czeizler, Virtanen, Rousu, Lähdesmäki, Penttilä, Jäntti, and Mojzita 2016].

Alternate strategies to enable similar regulatory control have focused primarily on modifying the promoter regions for a gene of interest, changing the inducing molecule, or using tunable CRISPR-based transcription factors (crisprTFs). Examples include Blazek *et al.* where a large number of native yeast promoters were used as the basis for a hybrid promoter that split the promoter into intact core regions and altered upstream activation sequences [Blazeck, Garg, Reed, and Alper 2012], and a similar, but broader, approach that altered both the upstream regions of native promoters as well as the corresponding synthetic DBDs using zinc finger motifs [Khalil, Lu, Bashor, Ramirez, Pyenson, Joung, and Collins 2012]. In addition to galactose and estradiol regulated promoters, tetracycline-inducible and camphor-repressible versions have been created to regulate gene expression [Garí, Piedrafita, Aldea, and Herrero 1997; Ikushima, Zhao, and Boeke 2015; Cuperus, Lo, Shumaker, Proctor, and Fields 2015]. dCas9 has also been used as a RNA-guided scaffold to recruit different protein effectors, thereby resulting in gene modulation [Gilbert et al. 2013]. Targeting crisprTFs to sequences upstream of TATA boxes resulted in gene activation that was further enhanced through addition of multiple operator sites [Farzadfard, Perli, and Lu 2013].

In this study, we used the LexA DBD, fused with the human estrogen binding domain (hEF) and the viral activator domain, VP16 as the synthetic-chimeric TF. Then, by using a combination of operator sequences in promoter scaffolds of native *S. cerevisiae* promoters, we aimed to generate a series of promoter combinations that, when coupled to the same TF, can be used to modulate gene expression to different extents using the same concentration of the inducer molecule (Figure 1). The potential of such a promoter library would be to induce multiple genes to discrete desired levels, with the same synthetic TF and a small inducer molecule added to the culture medium. Using a

combination of the j5 DNA assembly design software [Hillson, Rosengarten, and Keasling 2012] and the PR-PR laboratory automation platform [Linshiz, Stawski, Poust, Bi, Keasling, and Hillson 2013; Linshiz, Stawski, Goyal, Bi, Poust, Sharma, Mutalik, Keasling, and Hillson 2014], both developed at the Joint BioEnergy Institute (JBEI), and the gene synthesis capability at the Joint Genome Institute (JGI), a library of 240 promoter sequences were designed, 154 constructed and tested. The profiles of these regulatory systems are described in this report.

Materials & Methods

Strains and media:

Escherichia coli and *S. cerevisiae* strains, along with their associated information (including annotated sequence files), and sequences of all plasmids constructed are provided through the JBEI public registry [Ham, Dmytriv, Plahar, Chen, Hillson, and Keasling 2012] (https://public-registry.jbei.org/folders/277) and in Supplementary Table S1.

To produce ZDy1, the construct P_{ADH1}-LexA-hER-VP16 was integrated into *S. cerevisiae* BY4741 {MATa; *his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*} *S. cerevisiae* strain at locus YPRCΔ15 [Reider Apel et al. 2017; Flagfeldt, Siewers, Huang, and Nielsen 2009]. A complete description of this strain is available at https://public-registry.jbei.org/entry/9623. ZDy1 was transformed with promoter-reporter library plasmids using the conventional lithium acetate method [Gietz and Woods 2002], modified for large-scale transformation.

Experiments were conducted in synthetic defined (SD, 0.67% (w/v) yeast nitrogen base without amino acids (VWR International), 0.2% (w/v) complete supplement mixture w/o yeast nitrogen base (Sunrise Science Products) or standard rich media (YP, 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone) with 1% or 2% (w/ v) sugar. *E. coli* DH10b, used for cloning and plasmid amplification, were grown in LB supplemented with 100 µg/ml carbenicillin.

Design and construction of synthetic promoters:

Designs for the hybrid promoters were constructed using the j5 program [Hillson, Rosengarten, and Keasling 2012], by entering 1, 2 or 3 operator elements followed by the basal promoters. The number of possible combinatorial variants was reduced by specifying Eugene rules [Bilitchenko, Liu, Cheung, Weeding, Xia, Leguia, Anderson, and Densmore 2011] so that

only one type of operator sequence was present in any given design. The j5 software generated sequences for 240 hybrid promoters resulting from a combination of 3 operator configurations x 4 operator sequences x 10 basal promoters x 2 promoter lengths. Sequences of all hybrid promoter parts are listed in Supplementary Table S2.

For each hybrid promoter, oligomers for DNA synthesis were designed using the GeneDesign suite [Richardson, Nunley, Yarrington, Boeke, and Bader 2010], however, for 60 promoters the resulting sequences were deemed too difficult to synthesize due to the presence of multiple DNA synthesis constraint violations [Oberortner, Cheng, Hillson, and Deutsch 2017] and were abandoned. For the remaining 180 promoters, ultramer oligonucleotides were obtained from Integrated DNA Technologies (IDT; Coralville, IA), and DNA synthesis was performed using a 2-step PCR [Reisinger, Patel, and Santi 2006] method as described in [Heins et al. 2014]. Assembled fragments were cloned into the pRS426-yeGFP vector by chew-back cloning and transformed into *E. coli* TOP10 cells. Plating and picking was performed using a QPix 400 system (Molecular Devices). Eight colonies per construct were sequenced verified using PACBIO RSII system (Pacific Biosciences), and variant calling was performed using the GATK software package[McKenna et al. 2010]. One hundred and fifty-four successful constructs were recovered from *E. coli* and used to transform ZDy1 strains.

Promoter characterization:

Growth and fluorescence measurements in the presence of estradiol were conducted in 96-well plates on a Synergy H4 plate reader equipped with a Bio-Stack 3 Microplate Stacker (BioTek, Winooski, VT, USA).

Selected yeast clones harboring the promoter reporter constructs were grown overnight in 24-well plates in YNB, 1% dextrose, CSM –Ura medium. Cells were diluted into 8 mL of the same medium in 6-well plates and allowed to grow until reaching an OD_{600} of ~0.2-0.3. Cells were then inoculated into the final 96-well plates for promoter characterization: 100 µL of each clone was inoculated into each of 24 wells, so that six estradiol concentrations (0, 1, 5, 10, 50 and 100 nM) could be tested in technical quadruplicate. Additionally, this procedure was repeated for three biological clones of each promoter-reporter construct. Estradiol was added individually to each well at the specified concentrations just prior to the start of the experiment. Plates were covered with breathable adhesive plate seals (Thermo, NY, USA).

Prior to growth, plates were incubated at 23 °C, without shaking. To measure growth each plate was cycled by shaking for 30 seconds to resuspend the cells and aerate the culture, and the fluorescence (excitation: 485 ± 20 nm; emission: 528 ± 20nm) and OD_{600} was immediately acquired. Each plate was read every 30 minutes, and the cycling was repeated 72 times for a total run time of approximately 36 h.

Data analysis and methods to generate promoter profile plots.

The plate reader data was normalized as follows: 1) the baseline fluorescence of each well was normalized by setting the average of the first five time points after the first two to zero (the first two time points often gave artificially high fluorescence readings and so were ignored), 2) the background fluorescence was measured in a nonfluorescent BY4741 wildtype strain at each time point was subtracted from the readings of each strain in the same condition to eliminate the contributions of autofluorescence to overall fluorescence readings. Analysis of variance and regression analyses were conducted using R (R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (URL http://www.R-project.org/). Data used for these plots are provided in the supplementary section.

Results & Discussion

Design of Hybrid promoters and Synthetic-Chimeric TF

Since we wanted to generate a set of novel regulatory elements to enable the precise control of different genes and pathways using the same TF, the key variable in our study were the chimeric promoter sequences. We selected upstream sequences from 10 different native *S. cerevisiae* genes to act as basal promoter scaffolds: *GAL1p, LEU2p, SPO13p, TEF1p, HHF2p, GCN4p, CUP1p, HEM13p, ZRT1p*, and *SSL1p*. Promoter scaffolds were chosen with hopes of representing both constitutive and inducible profiles at both low and high expression levels. Generally, 100 bp upstream of a gene is considered the core promoter sequence, which is the minimal stretch of DNA needed to initiate transcription, while 250 bp is thought to contain other cis-acting elements that are involved in transcriptional regulation [Butler and Kadonaga 2002; Lubliner, Regev, Lotan-Pompan, Edelheit, Weinberger, and Segal 2015; Lubliner, Keren, and Segal 2013]. We selected a shorter (100 bp) and a longer (250 bp) sequence length for each of these promoters to serve as the promoter scaffold.

We replaced the upstream operator region of each promoter with one of four different known LexA operator sequences: consensus, uvrA, umuDC, and colE1 [Brent and Ptashne 1985] using 1x, 2x, or 3x repeats of these different LexA binding sequences. In total, we designed 240 plasmid-based promoter constructs driving a yeGFP that served as the reporter. All 240 promoters can be controlled by the same chromosomally integrated synthetic-chimeric TF. Our hypothesis was that these promoters would induce the gene of interest, in this case yeGFP, to different levels and have different inducibility, two important parameters for the design of biological circuits.

Building the Promoter library

Out of an initial set of 240 hybrid promoters that were designed, 60 constructs contained DNA synthesis constraint violations that precluded their synthesis and were abandoned at the design stage. DNA synthesis constraints violations are commonly encountered in synthetic biology designs and can be particularly severe when designing regulatory sequences, as they cannot be easily removed by codon shuffling. A strategy to overcome this limitation is to design experiments with sufficient redundancy such that biological insights can be obtained with a subset of the data. Out the 180 promoters that could be synthesized, 154 were successfully constructed and cloned into the *E. coli – S. cerevisiae* shuttle vector (pRS426-yeGFP) in order to characterize their functional parameters. This set of 154 promoters covered most of the biological space that we wanted to capture for this experiment (Supplementary Figure S1).

Induction and gene regulation using the promoter library

In order to easily test a large number of promoter constructs, we chose to chromosomally integrate a native ADH1 promoter driven copy of the LexA-hER-vp16 TF for stable expression. A similar, plasmid expressed, GAL4 estrogen responsive hybrid TF was previously shown to activate reporter genes exclusively in the presence of estradiol [Louvion, Havaux-Copf, and Picard 1993]. Replacement of the GAL4 DBD with the heterologous LexA prevents binding to native GAL4 promoters, thereby minimizing cross-talk.

For the promoter series, with the exception of TEF1, HHF2 and GCN4, most combinations basal promoters in two lengths could be built with 1x, 2x or 3x repeats of the four operator sequences. The plasmids with these hybrid promoter:yeGFP cassettes were transformed into the yeast strain with the chromosomally encoded synthetic TF. Estradiol concentrations from 0 – 100 nM were tested for each variant in the promoter library and a profile was generated for each strain. The complete set of plots for all hybrid promoters is provided as supplementary materials (Supplementary Fig S2). While good inducibility was observed from 5 – 50 nM of the inducer, 10 nM Estradiol was chosen as the inducer concentration that shows the maximal dynamic range for the promoter series (Figure 2).

Some studies have reported toxicity of VP16 and LexA-hER-VP16 in the presence of high levels of inducer [Garí, Piedrafita, Aldea, and Herrero 1997; McIsaac, Silverman, McClean, Gibney, Macinskas, Hickman, Petti, and Botstein 2011]. Both studies that reported toxicity did not observe growth inhibition in constructs with centromeric plasmids or chromosomally integrated versions with low levels of inducer. Consistent with this, with our experimental setup, we did not observe major toxicity at inducer concentrations of 10 nM or less, possibly due to the use of a chromosomally integrated ADH1 driven synthetic TF in combination with a hybrid promoter containing plasmid.

The parameters of interest that we quantified for each promoter were the inducibility and response, measured in terms of the fold change in induction from 0-100 nM and the maximal level of induction at 10 nM respectively. Analysis of variance was performed to statistically confirm the impact of each parameter and assess significance in the dataset (Figure 3). Overall, the promoter set with short upstream regions (100 bp) were significantly ($P<0.001$) less responsive and did not display wide range in either maximum fluorescence or inducibility regardless of promoter and operator sequences (Supplementary Figure S1, Figure 3: left panels). While only 20% of yeast genes contain TATA elements, a genome-wide study of their locations revealed that some genes contain TATA boxes further upstream than 100bp before the beginning of translation [Basehoar, Zanton, and Pugh 2004]. Therefore, it is possible that some of the short promoter scaffolds lack activity due to absence of TATA boxes or similar TATA protein-binding elements. For future design improvements, the lengths of the shorter promoter scaffolds could be individually customized to include functional TATA elements, which could result in better inducibility and range of induction.

Therefore, we focused our analysis on the set of promoters constructed using the longer promoter regions (250 bp). For this set, 84 total designs were constructed and tested. The main parameters, inducibility (fold change in fluorescence with the addition of estradiol) and response (maximal induction of GFP) displayed by the hybrid promoter library are summarized in Figure 4. Of the promoters tested, GAL1 and SPO13 showed the greatest inducibility. The GAL1 promoter also produced highest fluorescence (response), with the 3x uvrA GAL1 construct being the highest in our entire data set. A subset of promoters, such as HEM13 and ZRT1 with the uvrA and umuDC operators, gave very high fluorescence but at a constitutive level, that is: high response but low inducibility. Conversely, the SSL1 promoter with the uvrA and umuDC operators, and the LEU2 promoter with the umuDC operator showed low fluorescence at constitutive levels: that is both low response and low inducibility. Among the operator sequences, uvrA displayed significantly higher ($P<0.05$) inducibility than the consensus and other operator sequences (Figure 3: right panels), without changing max expression level for each promoter scaffold. Design improvements can also be envisioned for the longer promoters in this study. Domains in the promoter regions such as the upstream activating sequence (UAS) could be altered and optimized especially in the cases where the promoters were not highly inducible. The hybrid promoters based on GAL1 and SPO3 showed the greatest inducibility, but still contain some native UAS elements that may allow factors other than the inducer to influence the induction under a subset of growth conditions [Flick and Johnston 1990; Buckingham, H T Wang, Elder, McCarroll, Slater, and Esposito 1990].

Conclusions

Drawing inspiration from several reported synthetic transcription factors used in yeast, we have constructed a synthetic transcription factor that consists of a chimera of a bacterial DNA binding domain, a mammalian ligand binding domain, and a viral transcriptional activation domain. The gene encoding this transcription factor was integrated into the chromosome and expressed from a constitutive promoter. The synthetic promoters were constructed from the operators that bind the selected DNA binding domain and a native *S. cerevisiae* basal promoter that will recruit the basal transcription machinery. Transcription is activated by addition of the small molecule

ligand, estradiol, which binds the mammalian ligand-binding domain. We tested the hypothesis that a series of hybrid promoters with varying promoter scaffolds and operator regions will lead to different profiles in gene expression in response to a given level of the estradiol.

The synthetic promoters tested in our study in conjunction with the synthetic TF provide a suite of control systems we set out to compile; wherein in response to the same inducer and inducer concentration, the synthetic promoters can be used to achieve different gene expression profiles such as highly inducible and highly responsive, highly inducible but lowly responsive, constitutively on and highly active as well as constitutively on and lowly activity (Figure 2 & Figure 4). With additional characterization, these and other similar libraries allow a strain engineer to drive many genes and pathways in a cell to different levels using the same TF and same inducer added at a given level. This system has the potential to be useful for engineering heterologous enzymatic pathways in *S. cerevisiae* and for expressing multiple genes, or finding optimum levels for each gene product in a given pathway.

Acknowledgements

This work was part of the DOE Joint BioEnergy Institute (http://www.jbei.org) and part of the DOE Joint Genome Institute (http://jgi.doe.gov) supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, and was part of the Agile BioFoundry (http://agilebiofoundry.org) supported by the U.S. Department of Energy, Energy Efficiency and Renewable Energy, Bioenergy Technologies Office, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy. The United States Government retains and the publisher, by accepting the article for publication, acknowledges that the United States Government retains a nonexclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes. The Department of Energy will provide public access to these results of federally sponsored research in accordance with the DOE Public Access Plan (http://energy.gov/downloads/doe-public-access-plan).

Conflict of interest

J.D.K. has financial interests in Amyris, Lygos, Constructive Biology, Demetrix, and Napigen.

References

Alper H, Fischer C, Nevoigt E, Stephanopoulos G. 2005. Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. U.S.A.*, **102**: 12678–12683.

Basehoar AD, Zanton SJ, Pugh BF. 2004. Identification and distinct regulation of yeast TATA box-containing genes. *Cell*, **116**: 699–709.

Bilitchenko L, Liu A, Cheung S, Weeding E, Xia B, Leguia M, Anderson JC, Densmore D. 2011. Eugene – A Domain Specific Language for Specifying and Constraining Synthetic Biological Parts, Devices, and Systems. *PLoS ONE*, **6**: e18882.

Blazeck J, Garg R, Reed B, Alper HS. 2012. Controlling promoter strength and regulation in Saccharomyces cerevisiae using synthetic hybrid promoters. *Biotechnol. Bioeng.*, **109**: 2884–2895.

Brent R, Ptashne M. 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell*, **43**: 729–736.

Buckingham LE, Wang HT, Elder RT, McCarroll RM, Slater MR, Esposito RE. 1990. Nucleotide sequence and promoter analysis of SPO13, a meiosis-specific gene of Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U.S.A.*, **87**: 9406–9410.

Butler JEF, Kadonaga JT. 2002. The RNA polymerase II core promoter: a key component in the regulation of gene expression. *Genes Dev.*, **16**: 2583–2592.

Cuperus JT, Lo RS, Shumaker L, Proctor J, Fields S. 2015. A tetO Toolkit To Alter Expression of Genes in Saccharomyces cerevisiae. *ACS Synth Biol*, **4**: 842–852.

Farzadfard F, Perli SD, Lu TK. 2013. Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. *ACS Synth Biol*, **2**: 604–613.

Flagfeldt DB, Siewers V, Huang L, Nielsen J. 2009. Characterization of chromosomal

integration sites for heterologous gene expression in Saccharomyces cerevisiae. *Yeast*, **26**: 545–551.

Flick JS, Johnston M. 1990. Two systems of glucose repression of the GAL1 promoter in Saccharomyces cerevisiae. *Mol. Cell. Biol.*, **10**: 4757–4769.

Frederix M et al. 2014. Development of a native Escherichia coli induction system for ionic liquid tolerance. *PLoS ONE*, **9**: e101115.

Garí E, Piedrafita L, Aldea M, Herrero E. 1997. A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in Saccharomyces cerevisiae. *Yeast*, **13**: 837–848.

Gietz RD, Woods RA. 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Meth. Enzymol.*, **350**: 87–96.

Gilbert LA et al. 2013. CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes. *Cell*, **154**: 442–451.

Ham TS, Dmytriv Z, Plahar H, Chen J, Hillson NJ, Keasling JD. 2012. Design, implementation and practice of JBEI-ICE: an open source biological part registry platform and tools. *Nucleic Acids Res.*, **40**: e141.

Heins RA et al. 2014. Phylogenomically guided identification of industrially relevant GH1 β-glucosidases through DNA synthesis and nanostructure-initiator mass spectrometry. *ACS Chem. Biol.*, **9**: 2082–2091.

Hillson NJ, Rosengarten RD, Keasling JD. 2012. j5 DNA Assembly Design Automation Software. *ACS Synth Biol*, **1**: 14–21.

Ikushima S, Zhao Y, Boeke JD. 2015. Development of a Tightly Controlled Off Switch for Saccharomyces cerevisiae Regulated by Camphor, a Low-Cost Natural Product. *G3 (Bethesda)*, **5**: 1983–1990.

Khalil AS, Lu TK, Bashor CJ, Ramirez CL, Pyenson NC, Joung JK, Collins JJ. 2012. A synthetic biology framework for programming eukaryotic transcription functions. *Cell*, **150**: 647–658.

Lechner A, Brunk E, Keasling JD. 2016. The Need for Integrated Approaches in Metabolic Engineering. *Cold Spring Harb Perspect Biol*, **8**.

Lee ME, DeLoache WC, Cervantes B, Dueber JE. 2015. A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly. *ACS Synth Biol*, **4**: 975–986.

Linshiz G, Stawski N, Goyal G, Bi C, Poust S, Sharma M, Mutalik V, Keasling JD, Hillson NJ. 2014. PR-PR: cross-platform laboratory automation system. *ACS Synth Biol*, **3**: 515–524.

Linshiz G, Stawski N, Poust S, Bi C, Keasling JD, Hillson NJ. 2013. PaR-PaR Laboratory Automation Platform. *ACS Synth Biol*, **2**: 216–222.

Louvion JF, Havaux-Copf B, Picard D. 1993. Fusion of GAL4-VP16 to a steroid-binding domain provides a tool for gratuitous induction of galactose-responsive genes in yeast. *Gene*, **131**: 129–134.

Lubliner S, Keren L, Segal E. 2013. Sequence features of yeast and human core promoters that are predictive of maximal promoter activity. *Nucleic Acids Res.*, **41**: 5569–5581.

Lubliner S, Regev I, Lotan-Pompan M, Edelheit S, Weinberger A, Segal E. 2015. Core promoter sequence in yeast is a major determinant of expression level. *Genome Res.*, **25**: 1008–1017.

McIsaac RS, Gibney PA, Chandran SS, Benjamin KR, Botstein D. 2014. Synthetic biology tools for programming gene expression without nutritional perturbations in Saccharomyces cerevisiae. *Nucleic Acids Res.*, **42**: e48.

McIsaac RS, Oakes BL, Wang X, Dummit KA, Botstein D, Noyes MB. 2013. Synthetic gene expression perturbation systems with rapid, tunable, single-gene specificity in yeast. *Nucleic Acids Res.*, **41**: e57.

McIsaac RS, Silverman SJ, McClean MN, Gibney PA, Macinskas J, Hickman MJ, Petti AA, Botstein D. 2011. Fast-acting and nearly gratuitous induction of gene expression and protein depletion in Saccharomyces cerevisiae. *Mol. Biol. Cell*, **22**: 4447–4459.

McKenna A et al. 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.*, **20**: 1297–1303.

Mukhopadhyay A. 2015. Tolerance engineering in bacteria for the production of advanced biofuels and chemicals. *Trends in Microbiology*, **23**: 498–508.

Oberortner E, Cheng J-F, Hillson NJ, Deutsch S. 2017. Streamlining the Design-to-Build Transition with Build-Optimization Software Tools. *ACS Synth Biol*, **6**: 485–496.

Ottoz DSM, Rudolf F, Stelling J. 2014. Inducible, tightly regulated and growth condition-independent transcription factor in Saccharomyces cerevisiae. *Nucleic Acids Res.*, **42**: e130.

Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD. 2012. Microbial engineering for the production of advanced biofuels. *Nature*, **488**: 320–328.

Rantasalo A, Czeizler E, Virtanen R, Rousu J, Lähdesmäki H, Penttilä M, Jäntti J, Mojzita D. 2016. Synthetic Transcription Amplifier System for Orthogonal Control of Gene Expression in Saccharomyces cerevisiae Jennings B. (ed). *PLoS ONE*, **11**: e0148320.

Reider Apel A et al. 2017. A Cas9-based toolkit to program gene expression in Saccharomyces cerevisiae. *Nucleic Acids Res.*, **45**: 496–508.

Reider Apel A, Ouellet M, Szmidt-Middleton H, Keasling JD, Mukhopadhyay A. 2016. Evolved hexose transporter enhances xylose uptake and glucose/xylose co-utilization in Saccharomyces cerevisiae. *Scientific Reports*, **6**: 19512 EP–.

Reisinger SJ, Patel KG, Santi DV. 2006. Total synthesis of multi-kilobase DNA sequences from oligonucleotides. *Nat Protoc*, **1**: 2596–2603.

Richardson SM, Nunley PW, Yarrington RM, Boeke JD, Bader JS. 2010. GeneDesign 3.0 is an updated synthetic biology toolkit. *Nucleic Acids Res.*, **38**: 2603–2606.

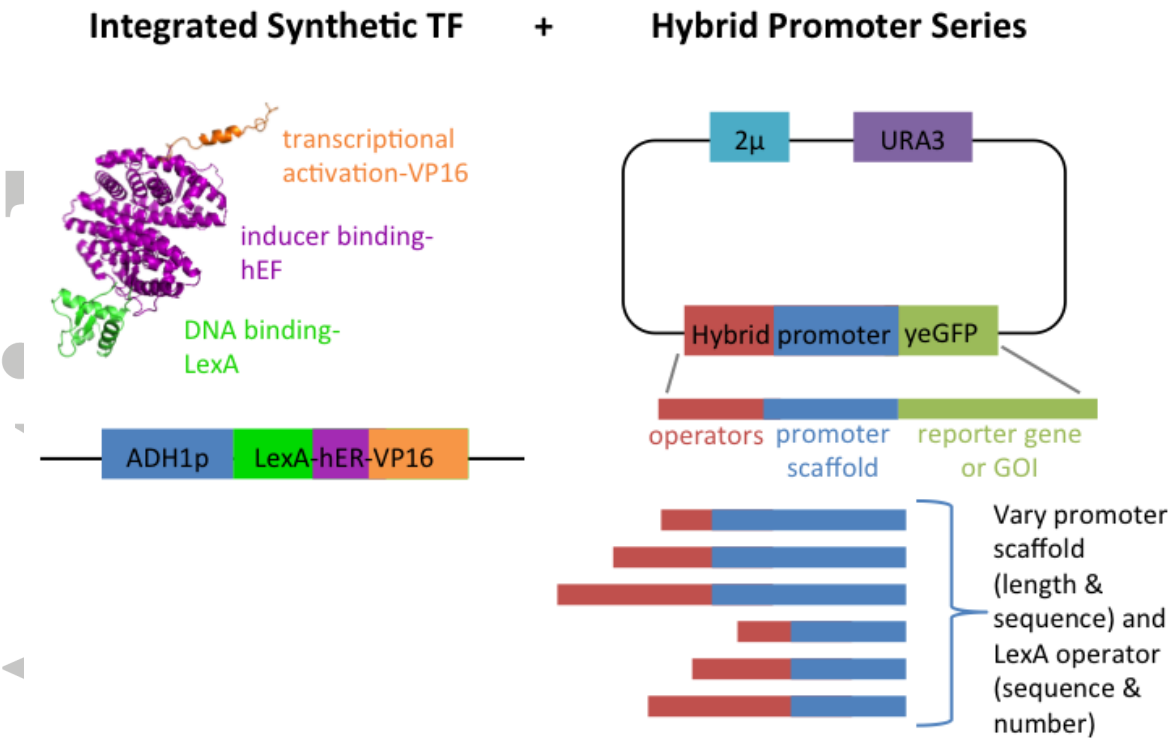

Figure 1. Design of modular TF and hybrid promoter strains. Schematic of our two-part control system where we, Left: constructed a strain (ZyD1) in which our hybrid transcription factor (*ADH1p*-LexA-hER-VP16) was integrated into locus YPRCΔ15 for stable expression. Right: A series of hybrid promoters were constructed with promoter scaffolds of varying lengths (100 bp or 250 bp) and identities (*GAL1p, LEU2p, SPO13p, TEF1p, HHF2p, GCN4p, CUP1p, HEM13p, ZRT1p*, and *SSL1p),* that were paired with one to three copies of each of four different operator sequences (consensus, uvrA, umuDC, and colE1). These 154 promoter combinations were expressed from a pRS426 plasmid and transformed into ZyD1 for analysis.

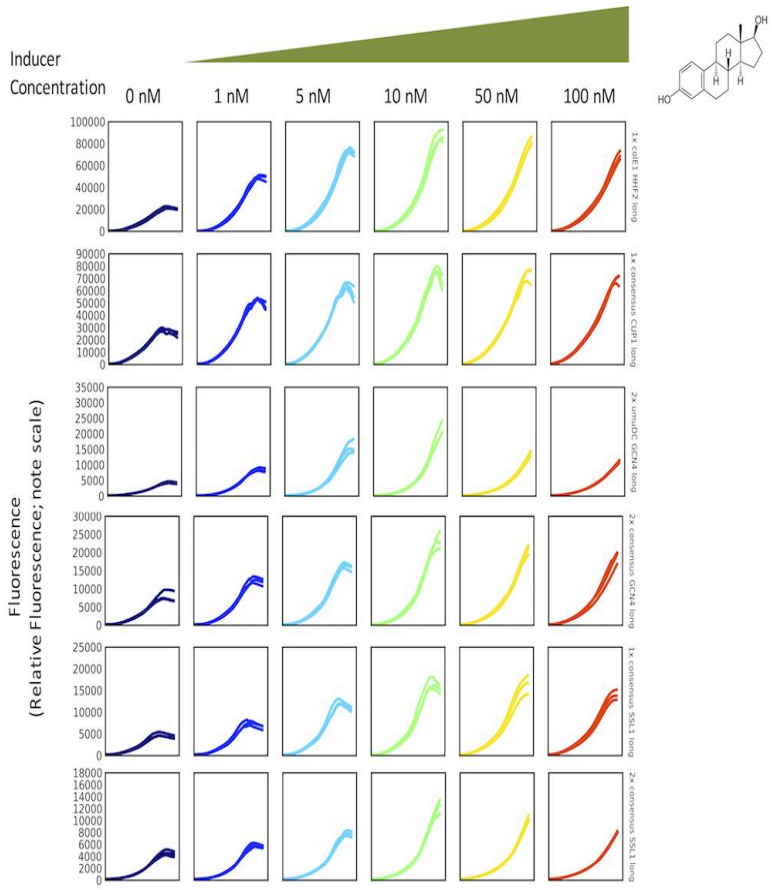

Figure 2. Subset of plots showing interesting hybrid promoter candidates. Florescence output from a subset of yeGFP expressing hybrid promoter strains (promoter names are labeled along right hand side) induced at various estradiol concentrations (0, 1, 5, 10, 50 and 100 nM) over a 36-hour time course. The plots show the range in maximum levels to which the promoters can be used for protein expression using the same level of estradiol and the range in inducibility. For the complete set of plots see **Figure S2**.

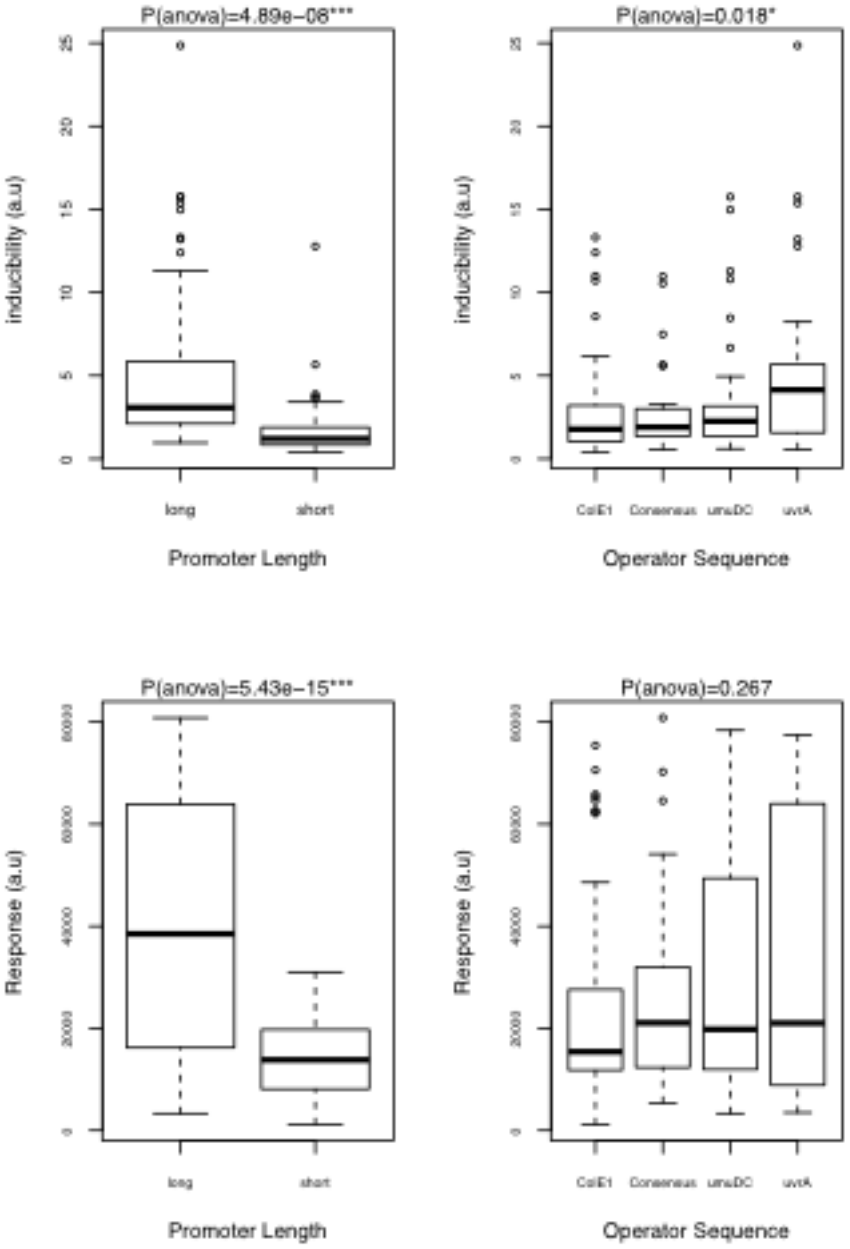

Figure 3. Parameters influencing promoter performance. Analysis of variance was conducted using R to establish inducibility fold (top) and max expression (bottom) significance for the dataset. P value significance: *<0.05, ***<0.001.

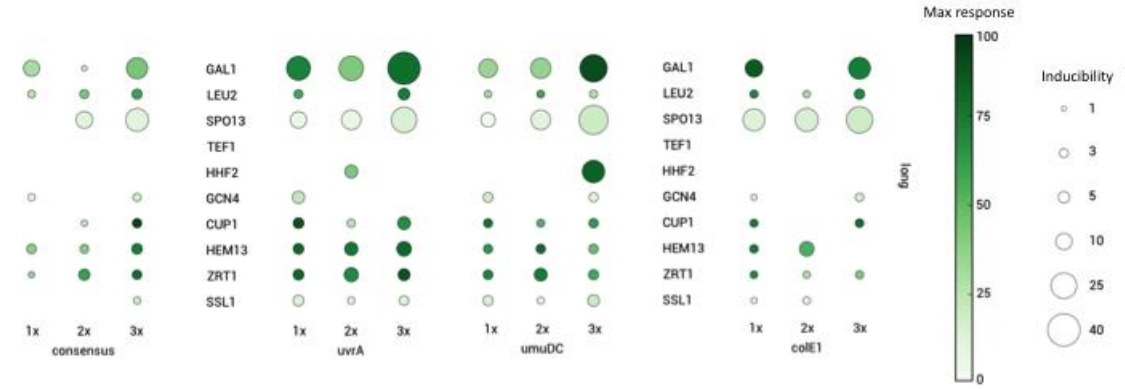

Figure 4. Long promoter scaffolds display a range of inducibility and responsiveness. Bubble plots showing inducibility fold (size of bubble) and maximum responsiveness level (color of bubble) of yeGFP expression for each of the 250 bp (long) constructed hybrid promoters.