



Systematic Transfer of Prokaryotic Sensors and Circuits to Mammalian Cells

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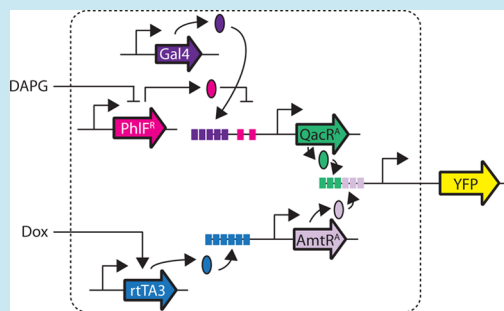
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S Supporting Information

ABSTRACT: Prokaryotic regulatory proteins respond to diverse signals and represent a rich resource for building synthetic sensors and circuits. The TetR family contains $>10^5$ members that use a simple mechanism to respond to stimuli and bind distinct DNA operators. We present a platform that enables the transfer of these regulators to mammalian cells, which is demonstrated using human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells. The repressors are modified to include nuclear localization signals (NLS) and responsive promoters are built by incorporating multiple operators. Activators are also constructed by modifying the protein to include a VP16 domain. Together, this approach yields 15 new regulators that demonstrate 19- to 551-fold induction and retain both the low levels of crosstalk in DNA binding specificity observed between the parent regulators in *Escherichia coli*, as well as their dynamic range of activity. By taking advantage of the DAPG small molecule sensing mediated by the PhlF repressor, we introduce a new inducible system with 50-fold induction and a threshold of $0.9 \mu\text{M}$ DAPG, which is comparable to the classic Dox-induced TetR system. A set of NOT gates is constructed from the new repressors and their response function quantified. Finally, the Dox- and DAPG-inducible systems and two new activators are used to build a synthetic enhancer (fuzzy AND gate), requiring the coordination of 5 transcription factors organized into two layers. This work introduces a generic approach for the development of mammalian genetic sensors and circuits to populate a toolbox that can be applied to diverse applications from biomanufacturing to living therapeutics.

KEYWORDS: mammalian synthetic biology, systems biology, eukaryote, inducible system, 2,4-diacetylphloroglucinol (DAPG)



Realizing the potential of engineering mammalian cells requires the predictable construction of synthetic sensors and circuits. In the clinic, cell-based therapies could function to integrate physiological markers, migrate to a disease location, and execute a multistep treatment.^{1–4} Cells involved in the manufacturing of biologics, such as the workhorse Chinese hamster ovary (CHO) cell line, could be engineered to respond to inducers that stage a multistep production process.^{5–7} Other applications include the programmable spatial organization needed for artificial organs and regenerative medicine, responsive living prosthetics (e.g., sensing blood glucose and controlling insulin production), and high-throughput drug screens based on readouts of cell state.^{1,8,9} These advanced applications require circuitry that encodes signal processing and control algorithms, the implementation of which requires more regulatory parts than are currently available.^{2,4} While the number of such parts for prokaryotes has exploded,¹⁰ there is currently a lag in building the analogous toolboxes for mammalian circuit design.¹¹

In prokaryotes, TetR transcriptional repressors constitute one of the most abundant and plastic family of regulators.^{12,13} These repressors consist of a single protein that contains both

small molecule sensing and DNA-binding domains. Over 200 000 TetR homologues have been sequenced that are representative of a wide range of sensing and DNA binding specificities. To date, sensing domains have been characterized that respond to >60 ligands, including antibiotics, metabolites, hormones, cell–cell signaling molecules, and metals.¹³ In the absence of its ligand, TetR forms a dimer that strongly binds to the TetR operator sequence (*tetO*). In the presence of ligand, the dimer is disrupted, TetR dissociates from the DNA, and gene expression is activated. The DNA-binding domains of different repressors bind unique 17–30 bp sequences, and it has been shown that these are highly orthogonal, with few off-target interactions between noncognate operators.¹⁴ Thus, this family provides a rich resource for mining ligand- and DNA-binding domains to build synthetic sensors and circuits.

The most common method for inducing mammalian gene expression is based on the TetR repressor, whose ligand is the

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antibiotic tetracycline.^{15,16} In mammalian cells, TetR can be used as both a repressor, or converted into an activator by fusing it to the transactivation domain from virion protein 16 of the *Herpes simplex* virus (VP16),¹⁷ which recruits RNA polymerase (RNAP). Multiple copies of *tetO* are placed upstream of the minimal CMV promoter (referred to as a Tetracycline Response Element or TRE), and in this fashion, reporter expression is activated when TetR is bound to the TRE, and becomes inactivated upon the addition of doxycycline (this system is referred to as “Tet-Off”). A “Tet-On” system has also been developed, whereby reporter expression is activated upon addition of doxycycline; this behavior is mediated by the reverse TetR transcription factor (rtTA).¹⁸ In both cases, the VP16 domain recruits RNA polymerase (RNAP) when TetR is bound to a synthetic, TRE-containing promoter. These switches typically have low basal expression, exhibit a large dynamic range (from 10 to several thousand-fold induction), and have been shown to function in a wide range of tissue culture systems, including embryonic stem cells,^{19,20} CHO,²¹ HEK,²² HeLa,²³ and MCF-7²⁴ cells, as well as in living animals.²⁵

Homologues of TetR have been used to build synthetic gene switches for various applications,^{2,26} and switches responding to other antibiotics, including erythromycin (MphR)²⁷ and pristinamycin (Pip)²⁸ have also been constructed. To expand upon the available inducible systems, sensors that respond to other small molecules (cumate, CymR²⁹) have been developed, including some that can be delivered to cells in gas form (acetaldehyde, AlcR;³⁰ 6-hydroxy-nicotine, HdnR³¹). Quorum sensing systems involved in cell–cell communication have been ported from *Streptomyces* (ScbR and SpbR),³² *Agrobacterium* (TraR),^{32,33} and *Vibrio fischeri* (LuxR).³⁴ These sensors have largely been developed for research purposes or in the context of a bioreactor. For clinical uses in patients, switches have been built that respond to nontoxic molecules, including amino acids (arginine, ArgR;³⁵ tryptophan, TrpR³⁶), food additives and metabolites (vanillic acid, VanR;³⁷ phloretin, TtgR³⁸), and vitamins (biotin,³⁹ BirA⁴⁰). Beyond cell cultures, many of these switches have been demonstrated to function in living animals, including mice.²⁷ In one compelling application, a uric acid (HucR) sensing circuit was constructed as part of a feedback mechanism to maintain blood urate homeostasis, the disruption of which can lead to gout.⁴¹ Furthermore, a sensor that reacts to the inactivation of antituberculosis compounds (EthR),⁴² which serves as an application for drug discovery, has also been constructed.

Of the many synthetic mammalian circuits that have been built using TetR and its homologues,⁴³ several of the resulting genetic switches and cascades based on these regulators exhibit ultrasensitivity and bistability.^{44–47} To build more sophisticated functions, logic operations such as inverters and 2-input Boolean gates have been layered together to generate feedforward circuits,⁴⁸ half adders (and subtractors),⁴⁹ 2-input decoders,⁵⁰ and a cell type classifier.⁵¹ Dynamic circuits have also been constructed, including time delays and oscillators.^{40,52–54} Furthermore, channels for cell–cell communication have also been developed where the sender signal (which consists of a metabolic pathway) produces the signaling molecule and the receiver acts as the signal sensor.^{40,55,56} To date, as many as 3 TetR homologues have been incorporated into a single mammalian circuit (tTA, PIP-KRAB, and E-KRAB^{57,58}), and in one case, up to 3 repressors (TtgR, TetR, and ScbR) were combined into a single protein.⁵⁹ However, the

construction of circuits that can perform more sophisticated signal processing operations will require a larger set of transcription factors that are orthogonal to one another, or in other words, that do not cross react with one another's DNA operators.

TetR and its homologues are not the only transcriptional regulators commonly used to construct genetic circuits, and several classes of transcription factors have modular DNA-binding domains that allow them to be programmed to target a specific nucleotide sequence.⁵⁹ This can be based on a combination of residues that bind to specific base pairs, as is the case for zinc finger proteins (ZFPs)⁶⁰ and transcription-activator-like effectors (TALEs).⁶¹ Similarly, the CRISPRi technique is based on the targeting of a catalytically inactive Cas9 protein to a specific DNA sequence through the use of a guide RNA.^{62,63} All of these systems can be moved into mammalian cells and retooled to function as activators or repressors by fusing VP16- or KRAB-like peptides, respectively,^{64–72} or by relying on steric hindrance of Cas9 alone.⁶⁷ However, it remains a challenge to add sensing capability to these DNA-binding domains. A generalizable approach (based on two-hybrid systems) has been to utilize two proteins whose dimerization is induced by a stimulus; such an approach has been used to build ZFPs and TALEs that respond to small molecules (e.g., rapamycin, hydroxytamoxifen, or RU486),^{73,74} hypoxia,⁷⁵ and light.^{76,77} The advantage of the TetR family is that a compact single protein has both the capability to sense a wide range of stimuli and transduce this to a DNA-binding event. Further, TetR and its homologues bind to small operator sequences with high specificity, which is desirable for promoter design but also comes at the cost of the inability to target them to arbitrary sequences.

Here, we present a systematic approach to retool a group of TetR-family repressors to operate as repressors and activators in mammalian cells. In previous work, we applied a part mining approach to build a set of 20 TetR homologues and characterized their orthogonality in *Escherichia coli*. Borrowing a strategy based off of designs used to convert TALEs into potent mammalian transcription factors,⁷⁸ we move 8 new TetR homologues (AmtR, BM3R1, ButR, IcaR, LmrA, McbR, PhlF, and QacR) into human embryonic kidney (HEK293) cells, retooled as 15 new activators and repressors. Remarkably, these transcription factors retain both the orthogonality and fold-change observed in prokaryotic cells.¹⁴ Ligand sensing is also preserved, and we use this to build a new inducible system, which we characterize in both HEK293 and CHO cells. We also measure their response functions as gates to aide in the construction of larger circuits. Collectively, this work demonstrates that prokaryotic part mining is an effective strategy for expanding the regulatory parts available for mammalian cell engineering.

■ RESULTS AND DISCUSSION

Functional Characterization of Retooled TetR Homologues in HEK293 Cells. In previous work, we used DNA synthesis to build a library of 73 TetR homologues,¹² of which we built responsive promoters for 20 in *E. coli*. The crosstalk within this subset was quantified by measuring the activity of 400 combinations of repressors and promoters. From these data, we selected a subset of 8 that are highly orthogonal to move into mammalian cells. The mammalian regulators were built using the complete protein sequence for each TetR homologue, where the corresponding gene was codon

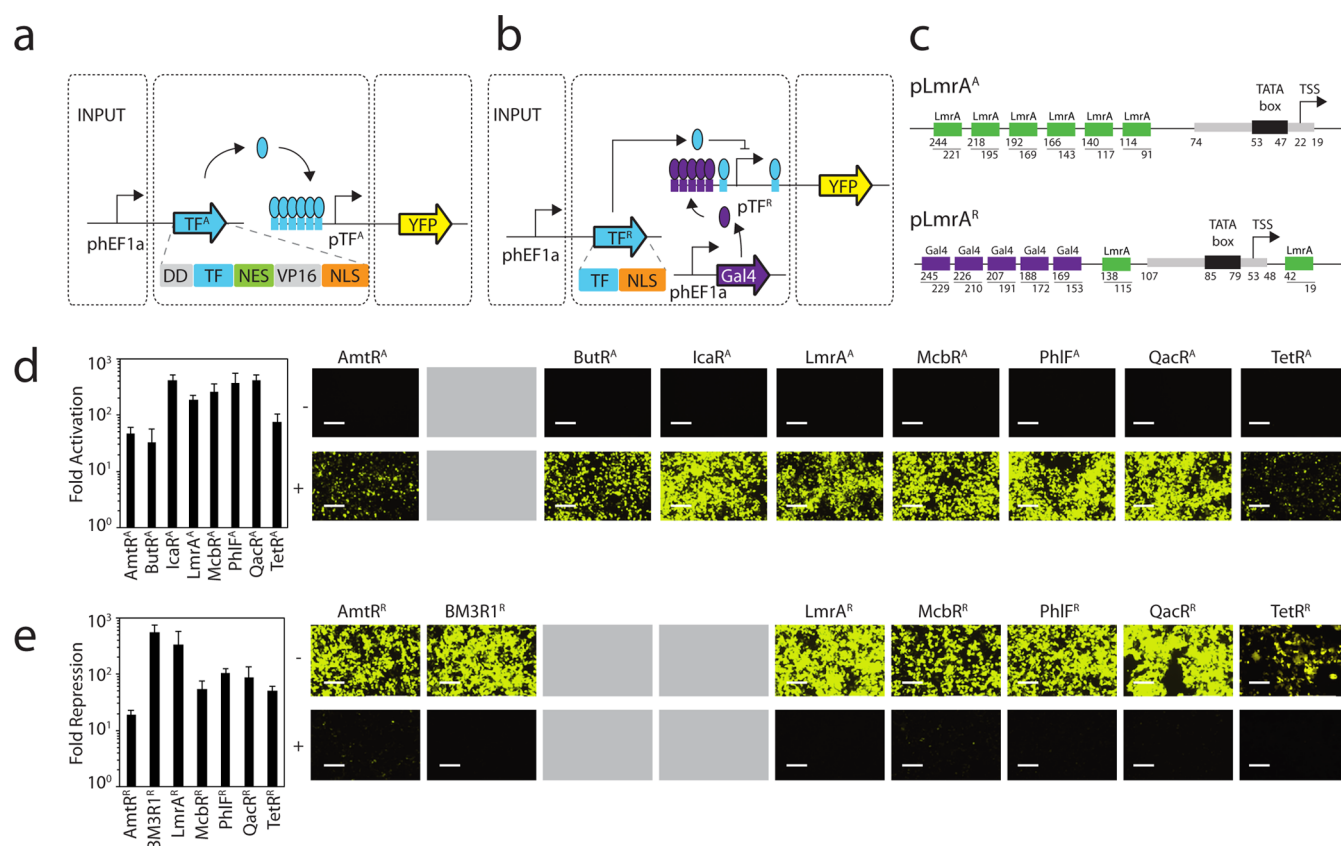


Figure 1. Design and characterization of synthetic transcription factors. (a) Expression of TF^A is controlled by the constitutive hEF1a promoter. Operator sequences are shown as boxes. pTF^A controls expression of the YFP output, which is activated by its cognate transcription factor. (b) The control system for TF^R is similar to part a except that Gal4-VP16 is constitutively expressed from a third plasmid. pTF^R controls expression of the YFP output, which is activated by Gal4-VP16 and repressed by its cognate transcription factor. (c) A detailed positional view of the activated ($pLmrA^A$, top) and repressed ($pLmrA^R$, bottom) *LmrA* promoters is illustrated. The $pLmrA^A$ promoter contains a minimal CMV promoter core with six upstream operators. The $pLmrA^R$ promoter consists of a minimal CMV promoter that is surrounded by two *LmrA* operators and five upstream Gal4 operators. The corresponding transcriptional start site (TSS) and TATA box are shown. (d) The function of the activators are shown and compared to the TetR activator ($TetR^A$). The fold-activation was calculated by comparing the average fluorescence in the presence of a plasmid encoding the activator (P-constitutive TF^A) with that obtained from the reporter plasmid (P- pTF^A reporter) in the absence of the P-constitutive TF^A plasmid. Cells were grown for 48 h post-transfection and assayed using flow cytometry (Methods). Representative histograms are shown in Supporting Information Figure 5. Microscopic images of cells transfected with the reporter only (–, top panel) or the cotransfected reporter and activator (+, bottom panel) are shown. BFP transfection controls are shown in Supporting Information Figure 6. (e) The function of the repressors are shown and compared to the TetR repressor ($TetR^R$). Fold-repression is calculated by comparing the average fluorescence in the presence and absence of the plasmid containing the repressor (P-constitutive TF^R). Microscopic images of cells transfected with the reporter and Gal4-VP16 (–, top panel) or the reporter, Gal4-VP16, and the repressor (+, bottom panel) are shown. Fluorescence histograms generated from the FITC-A geometric mean and BFP transfection control images are shown in Supporting Information Figures 7 and 8, respectively. In both parts d and e, the error bars were calculated based on the standard deviation of three independent experiments performed on different days. Cells are visualized using a YFP filter at 10× magnification, and images were taken 48 h post-transfection. The scale bars correspond to 400 μ m. Gray boxes indicate that a particular TetR homologue was converted into only an activator or repressor and the other version was either not built or is nonfunctional.

Table 1. Transcription Factor Operators and Inducer Molecules

TF	operator sequence	inducer molecule
AmtR	TTCTATCGATCTATAGATAAT	Gln K protein ¹¹²
BM3R1	CGGAATGAACGTTTCATCCG	pentobarbital ¹¹³
ButR	GTGTCACTTTGACAGCAGTGTCAC	unknown
IcaR	TTCACCTACCTTTCGTTAGGTTGT	gentamicin ¹¹⁴
LmrA	GATAATAGACCAGTCACATATTT	lincomycin ¹¹⁵
McbR	ATAGACTGGCCTGTCTA	L-methionine ¹¹⁶
PhlF	ATGATACGAAACGTACCGTATCGTTAAGGT	2,4-diacetylphloroglucinol ¹⁸⁵
QacR	TATAGACCGTGCATCGGTCTATA	plant alkaloids ¹¹⁷
TetR	TCCCTATCAGTGATAGA	doxycycline ¹¹⁸

optimized for expression in mammalian cells and resynthesized (Methods). Both activator (Figure 1a) and repressor (Figure 1b) versions were generated. Activators (TF^A) were built by

adding a destabilization domain,⁷⁹ a Nuclear Export signal (NES), a VP16 activation domain,⁸⁰ and a Nuclear Localization signal (NLS). Due to high levels of activation observed in the

absence of inducer, destabilization domains were added to the activator design. Only an NLS was added to build repressors (TF^{R}), which therefore rely on steric hindrance to achieve repression. The genes encoding TF^{A} and TF^{R} were placed under the control of the human elongation factor 1 α promoter (hEF1 α)⁸¹ and inserted into the pZDonor 1-GTW-2 plasmid⁸² (Supporting Information Figures 1 and 2).

Synthetic promoters were built for each of the mammalian transcription factors (Figure 1c). To generate activatable promoters, six copies of the cognate operator (Table 1) were inserted upstream of a minimal CMV promoter.^{78,83} A more complex promoter architecture is required in order to generate the repressible promoters, as the promoter itself must be activated in the absence of repressor. This behavior was achieved by designing a Gal4-VP16 activatable promoter. Specifically, each repressible promoter was designed to contain 5 Gal4 binding sequences upstream of a minimal CMV promoter, where Gal4-VP16 is constitutively expressed.^{67,78} The resulting promoter is rendered repressible by the inclusion of operators on either side of the CMV promoter. To measure activity, the promoters were placed upstream of a yellow fluorescent protein (YFP) coding sequence (Supporting Information Figure 3).⁸⁴ The transcription factors and reporters were maintained on separate pZDonor 1-GTW-2 plasmids.

The two-plasmid system containing the constitutively expressed transcription factor and the reporter were transiently transfected into HEK293 cells, as well as a single-plasmid transfection of the reporter alone. For the repressible system, a third plasmid was included from which Gal4-VP16 was expressed, and in all cases, a plasmid containing the constitutively expressed eBFP transfection control plasmid was included (Supporting Information Figure 4). Cells were then trypsinized 48 h post-transfection, and their fluorescence quantified using flow cytometry (Methods). The induction of the reporter in the presence and absence of the plasmid containing the constitutively expressed activator or repressor was then compared (Figure 1c and d, respectively, and Supporting Information Figures 5–8). Seven of the activators are highly functional and demonstrate an average of 225-fold activation (ranging from 33- to 416-fold). For comparison, an activator based on TetR is able to achieve 75-fold activation. In addition, six new repressors were obtained with an average of 172-fold repression (ranging from 18- to 551-fold). These levels of repression are comparable to the 50-fold repression that is achieved by TetR^R. While most of the TetR homologues could be systematically converted into both repressors and activators, for some only a single variant was found to be both functional and robust (BM3R1^R, ButR^A, and IcaR^A).

The division of transcription factors and reporters on separate plasmids facilitates the rapid measurement of crosstalk between noncognate pairs, and all combinations of reporters and transcription factors were cotransfected into HEK293 cells. The activators are largely orthogonal, with the exception of a few cross-reactions (Figure 2a and Supporting Information Figure 9). Notably, LmrA^A activates pQacR^A, and LmrA^A and QacR^A both activate pMcbR^A. The repressors are also highly orthogonal, although there is some activity of LmrR^R against pMcbR^R and pQacR^R (Figure 2b and Supporting Information Figure 10). Interestingly, the off-target interactions observed here are not present in the *E. coli* system.¹⁴ This may be due to changes in the expression level of the transcription factors, having multiple operators in the synthetic promoters, and/or

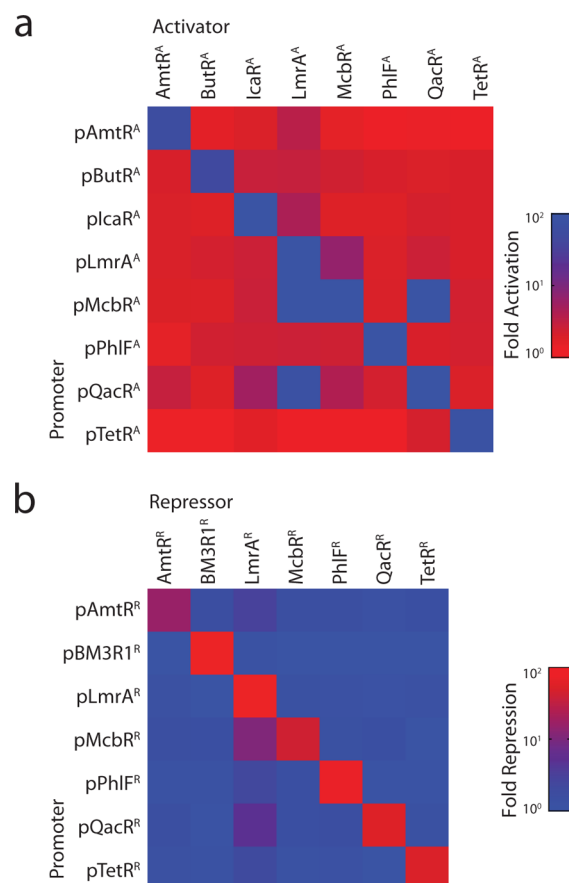


Figure 2. Orthogonality between synthetic transcription factors. (a) Crosstalk is shown between all combinations of activators and promoters. The fold-activation is calculated by dividing the average fluorescence of cells containing both the reporter and activator plasmids by the average fluorescence of cells only transfected with the reporter plasmid. Raw data underlying the matrix are shown in Supporting Information Figure 9, and data correspond to the average FITC-A geometric mean values from flow cytometry data collected from three independent transfections carried out on separate days. (b) Crosstalk is shown between all combinations of repressors and promoters. The fold-repression is calculated by dividing the average fluorescence of cells containing the reporter and Gal4-VP16 encoded plasmids by the fluorescence of cells transfected with plasmids encoding the reporter, Gal4-VP16, and cognate repressor. Raw data underlying the matrix are shown in Supporting Information Figure 10, and data correspond to the average FITC-A geometric mean values from flow cytometry data collected from three independent transfections carried out on separate days.

the ability of VP16 to recruit the transcriptional machinery even when delivered to a promoter at low affinity.

Construction of a DAPG-Inducible System. The TetR homologues that were selected for this study are associated with different classes of ligands, including metabolites, natural products, and plant alkaloids (Table 1). Similar to the doxycycline (Dox) induction of TetR in the Tet-On inducible system (Figure 3a),¹⁸ the PhIF repressor responds to 2,4-diacetylphloroglucinol (DAPG), which is a polyketide antibiotic produced by *Pseudomonas fluorescens* that has activity against plant pathogens (Figure 3b).^{85,86} DAPG has the potential to be a similarly useful inducible system, because it freely diffuses through eukaryotic membranes and can be purchased from chemical suppliers (Methods).

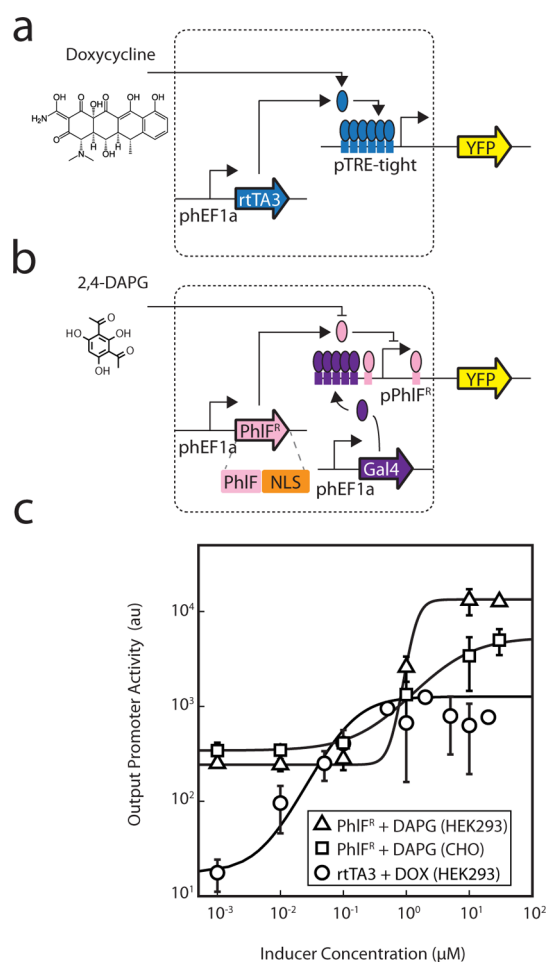


Figure 3. Characterization of the DAPG-inducible PhlF^R system. (a) The structure of doxycycline and the Tet-On inducible system, comprised of the rtTA3 regulator, are shown.¹¹¹ In this system, rtTA3 is constitutively expressed from the pHEF1a constitutive promoter and activates expression of its cognate promoter which contains 6 copies of the TetR operator sequence situated upstream of the minimal CMV promoter (referred to as pTRE-tight). The rtTA3 regulator binds to and activates expression from the pTRE-tight promoter in the presence of doxycycline. (b) The structure of DAPG and the PhlF^R inducible system are shown. In this system, PhlF^R is constitutively expressed from the pHEF1a promoter. The pPhlF^R output promoter is activated by Gal4-VP16, which is constitutively expressed by the pHEF1a promoter. PhlF^R binds to and represses expression from the pPhlF^R promoter in the absence of DAPG. (c) Induction of the Dox- and DAPG- inducible systems are compared and were measured in both HEK293 (Dox and DAPG systems) and CHO cells (DAPG system only). YFP fluorescence was measured after induction at [0, 0.01, 0.1, 1, 10, and 30 μM DAPG] or [0, 0.01, 0.05, 0.1, 0.5, 1, 2, 5, 10, 20 μM Dox]. The lines were fit to a Hill equation (Methods), the parameters for which are shown in Supporting Information Table 1. The data shown correspond to the average of three experiments from different transfections performed on different days, and error bars correspond to the standard deviation. Representative cytometry histograms for the three inducible systems are shown in Supporting Information Figure 11.

The inducibility of PhlF^R was tested by adding DAPG to transfected cells and measuring the response from the pPhlF^R reporter (Figure 3c; Supporting Information Figure 11 and Table 1). For cells supplemented with DAPG at the time of transfection, a drastic decrease in transfection efficiency was observed. To alleviate this decrease in transfection efficiency,

the inducer was instead added 6 h post transfection, and cells were incubated for 42 h (Methods). After induction, YFP expression was measured using flow cytometry. In HEK293 cells, the response yields a robust 54-fold induction with a notably ultrasensitive transition ($n = 4.7$ when fit to a Hill function), with a threshold (half-maximum) of 1 μM DAPG. This response is similar to what has been observed for the Tet-On inducible system, which has a similar dynamic range (70-fold) but a less cooperative transition ($n = 1.4$). However, greater leakiness is associated with the DAPG-inducible system (240 versus 18 au), and because of this, the response curve is shifted higher. We also tested the PhlF^R system in CHO cells, due to their importance in the manufacturing of biologics. This yielded a strong response, albeit with a lower dynamic range (15-fold) and less cooperative behavior ($n = 1.0$). The threshold of the switch is nearly identical among the two cell lines (5 μM DAPG in CHO cells), and the leakiness is also greater.

These discrepancies between the Dox- and DAPG-inducible systems can likely be attributed to their variable mechanisms used to control expression. For instance, our PhlF^R system is based on dual and opposing activities (activation by Gal4-VP16 and repression by PhlF). Such an architecture has been shown to result in ultrasensitivity.⁸⁷ In contrast, the Tet-On system relies on a more direct mechanism, whereby Dox induces rtTA3 binding to the promoter and subsequent activation of gene expression. Because of the large dynamic range associated with varying their inducer concentrations, both systems can be used to examine input-output relationships.

Measurement of 1-Input Response Functions. The response function of a gate captures how the output changes as a function of the input; for transcriptional gates, promoter activity serves as both the input and output. Our new repressors (TF^Rs) were used to build NOT gates,^{88,89} (which can be further converted into NOR gates by placing several upstream promoters in series).^{14,90} To deliver an input to the gate, the TRE-tight promoter (inducible by Dox) was used to drive expression of each TF^R (Figure 4a). The response function of this inducible system was measured separately in the same genetic context using a fluorescent reporter, where the output of each gate corresponds to the fluorescence of the TF^R-responsive promoter.

The response function for five repressors (McbR^R, PhlF^R, AmtR^R, BM3R1^R, and LmrA^R) was determined (Figure 4b and Supporting Information Figure 12). The average fluorescence was calculated by taking the mean YFP fluorescence from three experiments for each data point in the response curve; from these values, background fluorescence was subtracted, and the resulting output fluorescence values were converted into units of output promoter activity (this is done by separately measuring the activity of the various input promoters as a function of inducer). These values were used to generate a response function for each gate, where data were fit to a hill equation:

$$y = f(x) = y_{\min} + (y_{\max} - y_{\min}) \frac{K^n}{K^n + x^n} \quad (1)$$

where y is the activity of the output promoter, y_{\min} is the minimum output, y_{\max} is the maximum output, n is the Hill coefficient, and K is the threshold level of input where the output is half-maximal (Table 2). The output from the ON state (Dox = 1 nM) differs between each gate because it depends on the activity of the TF^R-responsive promoter, which

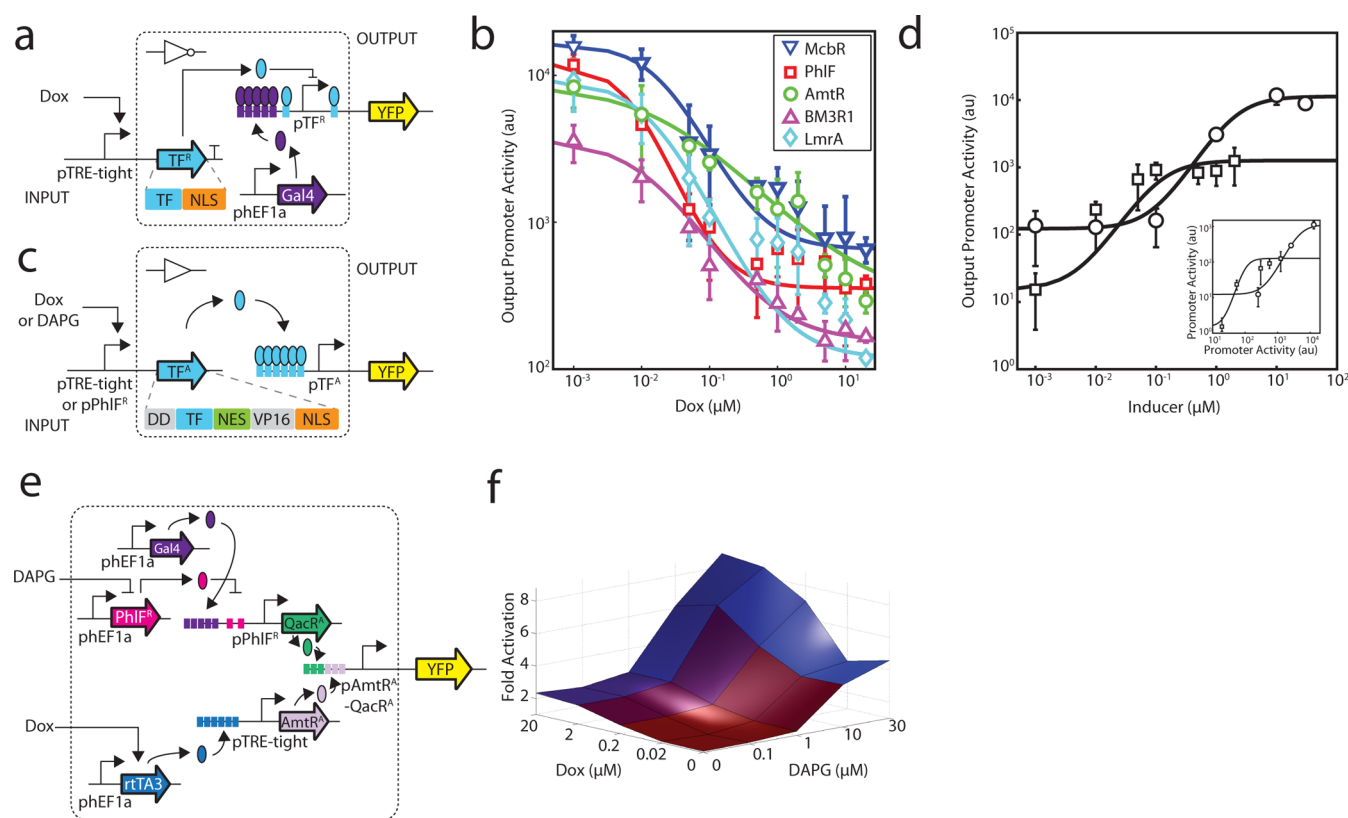


Figure 4. Gate and circuit response functions. (a) The Dox inducible system is used to characterize NOT gates. Symbols are as described in Figure 1. Expression of TF^R is controlled by the TRE-tight promoter, which is activated by the TetR activator ($rtTA3$) in the presence of Dox. Expression of the $rtTA3$ gene is controlled by the constitutive $hEF1a$ promoter. (b) The response of each NOT gate is shown: McbR (blue inverted triangles), PhlF (red squares), AmtR (green circles), BM3R1 (purple triangles), and LmrA (light blue diamonds). The expression of the fluorescent reporter from the output promoter (pTF^R) with respect to the induction of the input promoter ($pTRE-tight$) via Dox is shown. The average and standard deviation are plotted from three replicates from transfections performed on different days. Cytometry distributions corresponding to the FITC-A geometric mean of the 0, 0.5, and 5 μM induction points are shown in Supporting Information Figure 12 and fit parameters for each curve are listed in Table 2. (c) Two inducible systems (Dox or DAPG via $pPhlF^R$) are used to measure the response function of the buffer gates based on transcriptional activators (TF^A). (d) The response functions of the buffer gates are shown. The Dox-inducible system is used to characterize the $AmtR^A$ gate (circles) and the DAPG-inducible system is used to characterize the $QacR^A$ gate (squares). The inset shows the response as a function of input promoter activity ($pTRE-tight$ or $pPhlF^R$), rather than inducer concentration (Methods). Cytometry distributions corresponding to data for several induction points are shown in Supporting Information Figure 13 and fit parameters for each curve are listed in Table 3. (e) A schematic of the circuit that behaves as an enhancer is shown. The $pAmtR^A$ - $QacR^A$ promoter contains three upstream operators for each TF. (f) Enhancer fold activation of the output promoter ($pAmtR^A$ - $QacR^A$) is shown as a function of the two inducers, where inducer concentrations vary from 0 to 20 μM doxycycline and 0–30 μM DAPG. Activation is indicated in blue, and data correspond to average fluorescence values from three replicates collected on different days. Cytometry distributions and error bars are shown in Supporting Information Figures 15 and 16, respectively.

Table 2. NOT Gate Response Function Parameters

name	inducer	K^a	n	y_{max}^b	y_{min}^b	fold-change ^c
McbR ^R	Dox	0.13	1.22	1.6×10^4	6.6×10^2	24
PhlF ^R	Dox	0.05	1.50	1.2×10^4	3.6×10^2	33
AmtR ^R	Dox	0.17	1.07	8.4×10^4	2.9×10^2	28
BM3R1 ^R	Dox	0.09	1.07	3.6×10^4	1.7×10^2	21
LmrA ^R	Dox	0.12	1.46	9.3×10^4	1.2×10^2	77

^aThe threshold at which the NOT gate is at the half-maximum output, in μM doxycycline. ^bThe maximum and minimum levels of expression, in arbitrary units of YFP fluorescence. ^cThe fold-change is calculated by dividing the maximum average fluorescence (20 μM Dox) by the fluorescence of cells containing no inducer.

vary based on operator sequence. When maximally induced (Dox = 20 μM), all of the response functions converge on the same OFF state. The dynamic range is defined as the ON state divided by the OFF state, and this varies from 23- to 78-fold. All of the switches are noncooperative with a Hill coefficient

approaching unity ($n \approx 1$), which is expected because the promoters contain two noninteracting operators.

Buffer gates were also built based on the activators, which turn ON in response to induction from their input promoter. The response functions of the activators were measured either using the Dox-inducible $pTRE-tight$ promoter, as above, or the DAPG-inducible $pPhlF^R$ promoter from this study (Figure 4c). Using this approach, the response function of two activators ($AmtR^A$ and $QacR^A$) was determined following the same approach used for the NOT gates (Figure 4d and Supporting Information Figure 13). The data for each switch were fit using the following hill equation:

$$y = f(x) = y_{min} + (y_{max} - y_{min}) \frac{x^n}{K^n + x^n} \quad (2)$$

where the variables correspond to those used in equation 1 (parameters listed in Table 3).

When characterizing gates, it is useful to report the input and output promoters in the same units,^{14,91,92} which would allow

Table 3. Activator Response Function Parameters

name	inducer	K^a	n	γ_{\max}^b	γ_{\min}^b	fold-change ^c
AmtR ^A	Dox	0.1	3.00	1.3×10^3	15	82
QacR ^A	DAPG	4.6	1.89	1.1×10^4	122	91

^aThe threshold at which the buffer gate is at the half-maximum output, in μM doxycycline (for AmtR^A) or μM DAPG (for QacR^A). ^bThe maximum and minimum levels of expression, in arbitrary units of YFP fluorescence. ^cThe fold-change is calculated by dividing the maximum average fluorescence (20 μM Dox or 30 μM DAPG) by the fluorescence of cells containing no inducer.

the predictable connection of gates to form larger circuits (although this can be complicated by context effects^{93,94}). Yet the main challenge in doing so is that gates are typically measured using inducible systems and reported in terms of the concentration of the chemical inducer. When characterizing prokaryotic gates, we have separately measured the response of promoter output of the inducible system, and this information is used to build a response function that has the same units for the inputs and outputs.⁹⁵ Similarly, we could characterize the Dox- and DAPG-inducible systems and use this to renormalize the transfer functions of the NOT gates (Supporting Information Figure 15) and the switches (inset, Figure 4d). The hill coefficients for the inverters change after renormalization but are consistent with respect to one another. This variation can be attributed to the limited resolution in input promoter activity in our measurements that increases regression error. The characterized switches illustrated above act individually upon a promoter, yet composite promoters that respond to multiple transcription factors can also be constructed to provide tunable output control.

Signal Integration: Construction of an “Enhancer” Promoter That Responds to Two Activators. To generate a promoter capable of responding to combinations of input signals, operators for different transcription factors are typically combined into a single synthetic promoter. Similar approaches have been applied to build several classes of 2-input gates based on modified TetR homologues.⁵⁸ These circuits consist of a single activator (e.g., ScbR modified with VP16) and up to two repressors (e.g., Pip modified with KRAB). For example, a NOT IF gate was built by constructing a promoter that contains 8 upstream *ScbR* operators, followed by 3 *pir* operators in between and a minimum promoter motif. The resulting promoter is ON only in the presence of ScbR and in the absence of Pip. Here, we sought to determine whether our promoter architecture could integrate multiple positive regulators to converge on a single output.

To construct a hybrid promoter that is responsive to multiple transcription factors, we modified our initial architecture used to build synthetic promoters containing six upstream operators.⁷⁸ We postulated that this architecture could be altered to integrate signals from multiple TFs whose corresponding operators are present in different locations within the promoter. The full output of the promoter would not be achievable without induction of all of the TFs; thus, they would collectively enhance the activity of the promoter. The resulting circuit is not expected to function as an “AND gate” because each input increases activity toward the maximum. However, it does have features similar to fuzzy logic⁹⁶ and analog adder circuitry.⁹⁷

The integrating promoter was constructed by combining the operators for AmtR^A (3 downstream) and QacR^A (3 upstream,

Figure 4e). Specifically, AmtR^A expression is controlled by the Dox-inducible Tet-ON system, while QacR^A expression is controlled by the DAPG-inducible PhlF^R system (which also requires Gal4-VP16). Thus, the resulting circuit requires the control of 5 transcription factors carried on 7 distinct plasmids. All of the plasmids were cotransfected and the resulting YFP fluorescence measured using flow cytometry (Methods). The output was measured across varying concentrations of the two inducers (Dox and DAPG), and the resulting 25 data points were used to build a two-dimensional response function (Figure 4f and Supporting Information Figures 15 and 16). As expected, each inducible system is able to turn on the promoter independently, and the Dox-inducible system alone is able to induce the system 4.5-fold, while the DAPG-inducible system independently activates the system 8.5-fold. When both systems are maximally induced, the promoter is activated 19-fold. Thus, there is a near-perfect multiplicative effect between the induction of the two systems in isolation, compared to their collective impact on the promoter.

To gain insight into how transfection efficiency affects circuit performance, the fluorescence of the BFP-transfection control plasmid (a plasmid that constitutively expresses eBFP under the control of the hEF1a promoter) was used as a proxy for “copy number.” Since all plasmids are transfected in equal concentrations, it is expected that transfected cells contain the same relative amount of individual plasmids.⁹⁸ Therefore, cells with a higher “copy number” will have higher levels of eBFP expression, and a larger quantity of each plasmid. To assess the effect of “copy number” on circuit performance, cells were separated into 360 logarithmically spaced bins based on their BFP fluorescence, and the maximally inducing and noninducing conditions were compared for each bin (Supporting Information Figure 17). The “fuzzy” AND gate is quite robust, as it exhibits a consistent fold activation over a wide range of “copy numbers”.

Expanding the Mammalian Parts Toolbox and Beyond. The “fuzzy” AND gate demonstrated here, as well as the increased number of both sensors and circuits illustrated throughout, significantly expands upon the tools available for use in mammalian cells. We also systematically verify that these components exhibit minimal crosstalk and robust levels of fold change similar to their bacterial predecessors.¹⁴ Furthermore, we demonstrate their functionality across a variety of cell types including HEK293 and CHO cells. Finally, we reveal that these components can be combined in a single cell to coordinately fine-tune the expression of an individual output.

To obtain variable and specific output levels, we utilized a hybrid promoter architecture whereby two distinct TFs converge on a single promoter, through the inclusion of multiple copies of each TFs operator sequence. In mammalian cells, variable output levels are typically achieved through adjusting the number of transcriptional enhancer elements.⁹⁹ Enhancers integrate multiple signals *in vivo*, and act in *cis* to regulate transcriptional activity.¹⁰⁰ Not only the spacing but also the content of *cis*-regulatory elements have been shown to have a dramatic effect on biological processes (such as development) in eukaryotes.¹⁰¹ While enhancer elements alone can lack discernible activity, in concert with other elements they typically evoke robust expression patterns upon associated genes.¹⁰² Recent efforts have been dedicated to identifying mammalian enhancer elements, where naturally occurring sequences were assessed in parallel to identify the essential elements of transcriptional networks.^{103,104}

Although much work has been done to characterize the behavior and identity of naturally occurring enhancers, and to develop synthetic tools to control mammalian gene expression, issues persist in the implementation of such components toward broader applications. For example, the development of systems via transient transfection of tissue culture cells, and nonsite-specific integration make measurements difficult, and systems developed in this manner are not suited for clinical applications.³ Furthermore, it is known that enhancers exhibit negligible activity when transiently transfected but far more robust activity upon genomic integration.^{105–107} For these and other reasons, a safe harbor for genetic insertions should be developed, either through artificial chromosomes or designed integration sites.¹⁰⁸ Based on these findings, future efforts should focus upon rigorously characterizing the behavior of these and other components upon genomic integration. Delineating the contribution of integration site and copy number should be at the forefront of these efforts, as well as the engineering of epigenetic tools to ensure active expression of integrated circuitry. Breakthroughs in these areas will aid in the implementation of the tools presented here toward real world applications that span from living therapeutics to the production of complex pharmaceuticals.

METHODS

Cell Culture, Strains, and Media. *E. coli* strain DH10B [*F[−]mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15ΔlacX74 recA1 endA1 araΔ139 Δ(ara, leu)7697 galU galK λ-rpsL (StrR) nupG*] was used for cloning and to propagate DNA, except in the case where the propagated plasmids were used for Gateway cloning. In such cases, the *ccdB* Survival 2 T1R strain (Life Technologies, [*F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG fhuA::IS2*]) was used. The HEK293 (293FT) cell line was purchased from Invitrogen (product number R700-07), and CHO cells were obtained from ATCC (strain number CCL-61). HEK293 and CHO cells were cultured in high-glucose DMEM complete media (Dulbecco's modified Eagle's medium (DMEM), 4.5 g/L glucose, 0.045 units/mL of penicillin and 0.045 g/mL streptomycin and 10% FBS (Sigma)) at 37 °C, 100% humidity, and 5% CO₂. Doxycycline was purchased from Clontech (product number 631311), and 2,4-diacetylphloroglucinol (DAPG) was purchased from Santa Cruz Biotechnology (product number 206518).

Mammalian Genetic Parts. Supporting Information Table 3 contains all of the part sequences used in this study. Plasmid maps are provided in Supporting Information Figures 1–4. Prokaryotic repressor coding sequences were optimized for production in mammalian cells using multiparameter gene optimization methods and synthesized by Geneart.¹⁰⁹ The constitutive mammalian promoter (human elongation factor 1 alpha promoter, pHEF1a) was from pLEIGW, a gift from Ihor R. Lemischka. The rtTA3 coding sequence and the pTRE-tight promoter (containing the CMV minimal promoter) were amplified from pTRIPZ (GE Healthcare, product number RHS4743). Constitutively expressed BFP (pHEF1a-eBFP2) was used as a transfection control and was purchased from Addgene (plasmid 14891). The *rb glob PA* terminator was amplified from Addgene vector AAV-CAGGS-EGFP (plasmid 22212). In all cases, the reporter used corresponds to the Yellow Fluorescent Protein (eYFP),⁸⁴ and the DD-tag was purchased from Clontech (product number 632172).

Plasmids were constructed using a combination of GeneArt gene synthesis, Gateway cloning,¹¹⁰ and/or inverse PCR. Specifically, transcription factor coding sequences and their cognate promoters were synthesized into basic cloning vectors and were subcloned into expression or reporter vectors, respectively, via Gateway cloning. Hybrid promoters were constructed using inverse PCR to insert operator sequences upstream of the CMV minimal promoter within the reporter vector. In the case where inverse PCR was used to construct reporter vectors, whole plasmids were PCR amplified using Phusion DNA polymerase (NEB) along with multiple operator containing oligonucleotides. The resulting product was run on an agarose gel, extracted, and digested with DpnI. The blunted-ended, DpnI-digested product was phosphorylated (T4 Polynucleotide Kinase) and ligated (T4 DNA ligase) in a single reaction at room temperature, transformed into chemically competent DH10B cells, and plated on selective LB medium.

Transfection, Growth, and Processing of Cells.

HEK293 FT and CHO cells were transfected using the Attractene transfection reagent (Qiagen) as described in the manual with several modifications. Specifically, 100 ng of each plasmid was combined into the appropriate combinations in a total volume of 7 μL or less, and 60 μL Dulbecco's Modified Eagle Medium (DMEM) was added. To this mixture, 1.5 μL Attractene was added, and each sample was mixed by vortexing. The samples were incubated at room temperature for 10 min and then added to $\sim 8 \times 10^4$ cells in 0.5 mL DMEM that had been supplemented with penicillin, streptomycin, and amino acids (referred to as media complete) in a 24-well culture plate (Corning, product number 3473). For cells transfected with plasmids containing the pTRE-tight promoter, doxycycline was supplemented at the time of transfection. For cells containing plasmids harboring the DAPG-inducible system, DAPG was added 6 h post-transfection. Transfections were supplemented with 0.5 mL media complete 24 h post-transfection and doxycycline where appropriate. Cells were trypsinized 48 h post-transfection and subjected to flow cytometry (see below). Specifically, cells were trypsinized by aspirating the growth medium and applying 0.5 mL 0.25% trypsin-EDTA (Corning, product number 25-053) to adherent cells. Once cells were liberated from the plate, 2 mL media complete was added to each sample to halt trypsinization. Trypsinized cells were then spun down at 950 rpm for 10 min at 25 °C, the supernatant removed, and resuspended in 300 μL 1× phosphate buffered saline (PBS). From here, the trypsinized, PBS suspended cells were subjected to flow cytometry.

Flow Cytometry. Cells were analyzed by flow cytometry using a BD Biosciences LSRII flow cytometer. eBFP2 was measured using a 405 nm laser and a 450/50 filter, and eYFP with a 488 nm laser and a 530/30 filter. Cells were analyzed using FlowJo (TreeStar Inc., Ashland, OR), and populations were selected by gating out the background BFP signal of untransfected cells. Specifically, a gate was applied to encompass those cells that did not correspond to background (or where no signal was present on a BFP histogram of untransfected cells). The resulting gate was applied to all samples, to ensure that only cells expressing the eBFP2 transfection control were included in the analysis. Gated populations of >25 000 cells were used to calculate the geometric mean of the FITC-A fluorescence. When used to assay a promoter, this is referred to as the "Promoter Activity."

Fold Change and Circuit Copy Number Calculations.

All circuit plasmids were cotransfected with the transfection control marker, P-constitutive-eBFP. BFP-positive cells were separated into 360 logarithmically spaced bins based on raw fluorescence, referred to as the "Transfection Marker". Fold-activation was calculated by dividing FITC-A fluorescence values from fully induced cells (20 μ M DOX and 30 μ M DAPG) by uninduced cells within each bin.

Hill Equation Curve Fitting. Response curves parameters for all activators and repressors were calculated by fitting to their respective Hill equations (equations 1 and 2). For each input, average fluorescence values from biological triplicates (collected on different days) were fit to the appropriate form of the Hill equation. Nonlinear least-squares regression was used to determine values for the Hill coefficient (n) and dissociation constant (K), and to minimize the error between the fitted and actual values.

Calculation of Fold-Change. The fold-change was determined by dividing the background subtracted YFP fluorescence values for cells containing the reporter plasmid alone (P-pTF^R-reporter) by that of cells containing both the reporter and the transcription factor (either P-constitutive TF^R, P-TRE-tight/TF^R, or P-PhIF^R/TF^R) encoding plasmids, in the case of the repressors (where both transfections contained plasmids P-constitutive-Gal4-VP16 and P-constitutive-eBFP). For the activators, fold-change was calculated by taking the inverse of the equation used to calculate the fold-change for the repressors (where both transfections contained the P-constitutive-eBFP plasmid).

Microscope Imaging. Images were taken using an EVOS Digital inverted microscope (containing a 3MP color digital camera and LCD display). The excitation and emission wavelengths to obtain fluorescent images were as follows: 357 nm excitation 447 nm emission for eBFP, and 500 nm excitation 542 nm emission for eYFP. Images were taken at a 10 \times objective.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

B.C.S., A.G., and C.A.V. designed experiments, performed experiments, analyzed data, and wrote the manuscript. V.S. and L.W. designed and performed experiments. K.C., A.T., and J.C. designed experiments. R.W. designed experiments, analyzed data, and edited the manuscript.

Notes

The authors declare no competing financial interest.

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