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. 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. Other applications include the programmable spatial organization of synthetic 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)
antibiotic tetracycline. 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), 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). 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, CHO, HEK-293, HeLa, and MCF-7 cells, as well as in living animals.

Homologues of TetR have been used to build synthetic gene switches for various applications, and switches responding to other antibiotics, including erythromycin (MphR) and pristinamycin (Pip), have also been constructed. To expand upon the available inducible systems, sensors that respond to other small molecules (cumate, CymR) have been developed, including some that can be delivered to cells in gas form (acetalddehyde, AlcR; 6-hydroxy-nicotine, HdnoR). Quorum sensing systems involved in cell–cell communication have been ported from Streptomyces (ScbR and SpbR), Agrobacterium (TrAR), and Vibrio fischeri (LuxR). 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), tryptophan, TrpR, food additives and metabolites (vanillin acid, VanR, phloretin, TgR), and vitamins (biotin, BirA). Beyond cell cultures, many of these switches have been demonstrated to function in living animals, including mice. 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. Furthermore, a sensor that reacts to the inactivation of antituberculosis compounds (EthR), 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, several of the resulting genetic switches and cascades based on these regulators exhibit ultrasensitivity and bistability. To build more sophisticated functions, logic operations such as inverters and 2-input Boolean gates have been layered together to generate feedforward circuits, half adders (and subtractors), 2-input decoders, and a cell type classifier. Dynamic circuits have also been constructed, including time delays and oscillators. 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. To date, as many as 3 TetR homologues have been incorporated into a single mammalian circuit (tTA, PIP-KRAB, and E-KRAB), and in one case, up to 3 repressors (TgR, TetR, and ScbR) were combined into a single protein. 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. This can be based on a combination of residues that bind to specific base pairs, as is the case for zinc finger proteins (ZFPs), transcription-activator-like effectors (TALEs). 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. All of these systems can be moved into mammalian cells and retooled to function as repressors or activators by fusing VP16- or KRAB-like peptides, respectively, or by relying on steric hindrance of Cas9 alone. 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). Hypoxia, light, and the advantages 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, 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. 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 aid 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, 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...
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, a Nuclear Export signal (NES), a VP16 activation domain, and a Nuclear Localization signal (NLS). Due to high levels of activation observed in the presence of the activator, the pLmrAA promoter contains a minimal CMV promoter core with six upstream operators. The pLmrAR 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 illustrated. The function of the activators are shown and compared to the TetR activator (TetRA). The fold-activation was calculated by comparing the average fluorescence in the presence of a plasmid encoding the activator (P-constitutive TFA) with that obtained from the reporter plasmid (P-pTFA reporter) in the absence of the P-constitutive TFA 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. 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 μm. 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

<table>
<thead>
<tr>
<th>TF</th>
<th>operator sequence</th>
<th>inducer molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmtR</td>
<td>TTCTATCGATCTATAGATAAT</td>
<td>Gln K protein^112</td>
</tr>
<tr>
<td>BM3R</td>
<td>CGGAATGAACGGTCATTCCGG</td>
<td>penicillin^113</td>
</tr>
<tr>
<td>ButR</td>
<td>GTGTCACTTTGGACAGGCTGCTCAC</td>
<td>unknown</td>
</tr>
<tr>
<td>IcaR</td>
<td>TTTCCCTACCTGTTGGATTTAAAGTTGT</td>
<td>gentamicin^114</td>
</tr>
<tr>
<td>LmrA</td>
<td>GATAAAGCCGACGTACATATTT</td>
<td>lincomycin^115</td>
</tr>
<tr>
<td>McbR</td>
<td>ATAGACTGGGTCTGCTA</td>
<td>L-methionine^116</td>
</tr>
<tr>
<td>PhlF</td>
<td>ATGATACGAAAGCCCTGATCGTTAAAGGT</td>
<td>2,4-diacetylphloroglucin^85</td>
</tr>
<tr>
<td>QacR</td>
<td>TATAGACGGCCGACGTGCTTATAGA</td>
<td>plant alkaloids^117</td>
</tr>
<tr>
<td>TetR</td>
<td>TCCCTATCGAGTATAGA</td>
<td>doxycycline^118</td>
</tr>
</tbody>
</table>
The activators are largely orthogonal, with the exception of a few cross-reactions (Figure 2a and Supporting Information Figure 3). 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 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).

Constructions of 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, LmrRA activates PncAR and LmrRA and QacAR both activate McbAR. The repressors are also highly orthogonal, although there is some activity of LmrRA against McbAR and QacAR (Figure 2b and Supporting Information Figure 10). Interestingly, the off-target interactions observed here are not present in the E. coli system. This may be due to changes in the expression level of the transcription factors, having multiple operators in the synthetic promoters, and/or 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), 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). 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).
The inducibility of PhlFR was tested by adding DAPG to transfected cells and measuring the response from the pPhlFR 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 PhlFR 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 PhlFR 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. 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 (TPFks) were used to build NOT gates, which can be further converted into NOR gates by placing several upstream promoters in series. To deliver an input to the gate, the TRE-tight promoter (inducible by Dox) was used to drive expression of each TFK (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 TFK-responsive promoter.

The response function for five repressors (McbRR, PhlFR, AmtR, Bm3R1, and LmrA) 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:

\[ y = f(x) = y_{\text{min}} + \left( y_{\text{max}} - y_{\text{min}} \right) \frac{K^n}{K^n + x^n} \]  

where y is the activity of the output promoter, \( y_{\text{min}} \) is the minimum output, \( y_{\text{max}} \) 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 TFK-responsive promoter, which

Figure 3. Characterization of the DAPG-inducible PhlFR system. (a) The structure of doxycycline and the Tet-On inducible system, comprised of the rtTA3 regulator, are shown. 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 PhlFR inducible system are shown. In this system, PhlFR is constitutively expressed from the phEF1a promoter 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,
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 ≈ 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 pPhlFR promoter from this study (Figure 4c). Using this approach, the response function of two activators (AmtRA and QacRA) 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:

\[ y = y_{\min} + \left( y_{\max} - y_{\min} \right) \frac{x^n}{K^n + x^n} \]  

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 2. NOT Gate Response Function Parameters

<table>
<thead>
<tr>
<th>Name</th>
<th>Inducer</th>
<th>K</th>
<th>n</th>
<th>( y_{\text{max}} )</th>
<th>( y_{\text{min}} )</th>
<th>Fold-change</th>
</tr>
</thead>
<tbody>
<tr>
<td>McbR</td>
<td>Dox</td>
<td>0.13</td>
<td>1.22</td>
<td>1.6 \times 10^4</td>
<td>6.6 \times 10^2</td>
<td>24</td>
</tr>
<tr>
<td>PhiL</td>
<td>Dox</td>
<td>0.05</td>
<td>1.50</td>
<td>1.2 \times 10^4</td>
<td>3.6 \times 10^2</td>
<td>33</td>
</tr>
<tr>
<td>AmmR</td>
<td>Dox</td>
<td>0.17</td>
<td>1.07</td>
<td>8.4 \times 10^4</td>
<td>2.9 \times 10^2</td>
<td>28</td>
</tr>
<tr>
<td>BM3R</td>
<td>Dox</td>
<td>0.09</td>
<td>1.07</td>
<td>3.6 \times 10^4</td>
<td>1.7 \times 10^2</td>
<td>21</td>
</tr>
<tr>
<td>LmrA</td>
<td>Dox</td>
<td>0.12</td>
<td>1.46</td>
<td>9.3 \times 10^3</td>
<td>1.2 \times 10^2</td>
<td>77</td>
</tr>
</tbody>
</table>

\(a\) The threshold at which the NOT gate is at the half-maximum output, in μM doxycycline. \(b\) The maximum and minimum levels of expression, in arbitrary units of YFP fluorescence. \(c\) The fold-change is calculated by dividing the maximum average fluorescence (20 μM Dox) by the fluorescence of cells containing no inducer.
Table 3. Activator Response Function Parameters

<table>
<thead>
<tr>
<th>name</th>
<th>inducer</th>
<th>$K^a$</th>
<th>$n$</th>
<th>$y_{\max}^b$</th>
<th>$y_{\min}^b$</th>
<th>fold-change$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmtR$^a$</td>
<td>Dox</td>
<td>0.1</td>
<td>3.00</td>
<td>$1.3 \times 10^5$</td>
<td>15</td>
<td>82</td>
</tr>
<tr>
<td>QacR$^a$</td>
<td>DAPG</td>
<td>4.6</td>
<td>1.89</td>
<td>$1.1 \times 10^3$</td>
<td>122</td>
<td>91</td>
</tr>
</tbody>
</table>

$^a$ The threshold at which the buffer gate is at the half-maximum output, in $\mu$M doxycycline (for AmtR$^a$) or $\mu$M DAPG (for QacR$^a$). $^b$ The maximum and minimum levels of expression, in arbitrary units of YFP fluorescence. $^c$ The fold-change is calculated by dividing the maximum average fluorescence (20 $\mu$M Dox or 30 $\mu$M 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 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.$^{96}$ These circuits consist of a single activator (e.g., SbR 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 SbR operators, followed by 3 pir operators in between and a minimum promoter motif. The resulting promoter is ON only in the presence of SbR 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 logic$^{97}$ 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 PhlR 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. Furthermore, it is known that enhancers exhibit negligible activity when transiently transfected but far more robust activity upon genomic integration. For these and other reasons, a safe harbor for genetic insertions should be developed, either through artificial chromosomes or designed integration sites. 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-merBC) Φ80lacZΔM15ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ-ρspL (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 TIR strain (Life Technologies, [F− mcrA Δ(mrr-hsdRMS-merBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK ρspL (StrR) endA1 nupG [fhuA::IS2]) was used. The HEK293 (293FT) cell line was purchased from Invitrogen (product number 206518). 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 ~8 x 10^6 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 µL media complete 24 h post-transfection and doxycycline where appropriate. Cells were trypsinized 48 h post-transfection and subjected to flow cytometry (see below).

Plasmids were constructed using a combination of GeneArt gene synthesis, Gateway cloning 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 ~8 x 10^6 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 µL media complete 24 h post-transfection and doxycycline where appropriate. Cells were trypsinized 48 h post-transfection and subjected to flow cytometry (see below).

### 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 530/30 bandpass filter. Cells were trypsinized by aspirating the growth medium and applying 0.5 µL 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 1X phosphate buffered saline (PBS). From here, the trypsinized, PBS suspended cells were subjected to flow cytometry.
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 μM DOX and 30 μM 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 yellow fluorescent values for cells containing the reporter plasmid alone (P-pTFF-reporter) by that of cells containing both the reporter and the transcription factor (either P-constitutive TF, P-TRE-tight/TF, or P-PhlFR/TF encoding plasmids, in the 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 10X objective.

**REFERENCES**


Research Article


