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Tetracycline-Regulated Expression Implemented through Transcriptional Activation Combined with Proximal and Distal Repression

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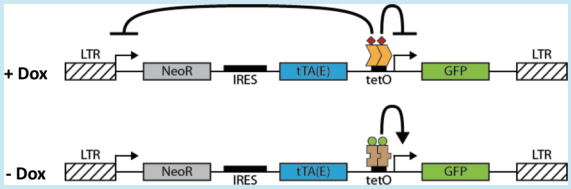
Supporting Information

ABSTRACT: Tetracycline-regulated expression systems are widely used to control ectopic gene expression in mammalian cells. However, background or "leaky" expression in the "off" state can limit applications that require control of expression at low levels. In this work we have engineered a tetracycline-regulated expression system with an improved range of control and lower background expression. To lower background expression without diminishing the controllable expression range, we designed a feed-forward scheme that repressed both expression of the gene of interest and the transcriptional activator. By using a tetracycline-responsive repressor that can modify chromatin and repress transcription over short and long distances, we were able to repress these two expression targets using a single tetracycline-responsive genetic element. This dual-targeting repressor/activation system demonstrated decreased background expression in its "off" state and a 25-fold range of expression in response to doxycycline. This study demonstrates that genetic circuits can be improved by leveraging trans-acting factors with long-range capabilities.



KEYWORDS: *tetracycline repressor, KRAB repressor, tetracycline-responsive transcriptional transactivator, inducible gene expression*

Tetracycline-regulated expression systems have been utilized to control gene expression in a wide range of biological systems and applications.[1−7] One basic system used in mammalian cells consists of a tetracycline-responsive transcription factor and a tetracycline-responsive promoter, which consists of tet-operator (tetO) sequences and a minimal transcriptional promoter. The tetracycline-responsive transcriptional activator (tTA) consists of a fusion of a DNA-binding domain, the tetracycline repressor (tetR) from *E. coli*, and a transcriptional activator domain, VP16 from the Herpes Simplex Virus.[8,9] Transcription is then controlled by adding or taking away tetracycline or the oft-used analogue doxycycline, which has higher specificity[10] and stability in solution.[11] When tetracycline or doxycycline is added, it binds to the tetR domain of the tTA and reduces the binding affinity between the tTA and the tet operator (tetO) sequences contained in the tetracycline-responsive promoter.[10,12] As a result, less activator is recruited to the promoter and gene expression is decreased or turned "off" (Figure 1A, left). Another commonly used synthetic transcription factor is the reverse tetracycline-responsive transcriptional activator (rtTA).[9,13] Like the tTA, the rtTA is a fusion between tetR and VP16, but here point mutations have been added to the tetR domain so that its DNA binding affinity increases upon addition of tetracycline or doxycycline. As a result, more activator is recruited, and gene expression is increased or turned "on".

With most inducible expression systems, there is considerable background expression in the "off" state.[1,2] While a low level of "leaky" expression is tolerable for many applications, it can become an issue when gene products are toxic or produce specific behavior at low expression levels. To reduce leaky expression, tetracycline-responsive repressors have been used in conjunction with the tetracycline-responsive activator.[14−16] With one engineered version of a tetracycline-responsive repressor, tetR has been fused to a trans-acting repression domain known as the Krüppel associated box (KRAB), a conserved repression domain found in many zinc finger proteins.[17] Unlike other repressors that bind to DNA and form a physical barrier to RNA polymerase (e.g., LacI of the lac operon), the KRAB-mediated repressor recruits factors that chemically modify histones (e.g., through deacetylation) to generate heterochromatin that does not support transcription. Additionally, unlike most of the passive, barrier-forming repressors, the KRAB-mediated repressors can act over long distances (2−3 kb reported) and repress transcription from multiple nearby promoters.[18]

In this study, we sought to utilize a synthetic KRAB repressor and a synthetic VP16 activator that both contain a tetR DNA-binding domain. The active form of the tetracycline repressor is a homodimer comprising two tetR monomers, and without dimerization it will not effectively bind to the tetO DNA sequences.[12] Furthermore, one cannot coexpress a tetracycline-responsive transcriptional repressor and activator with the same, compatible tetR dimerization domains. This would lead to heterodimers that would simultaneously activate and repress

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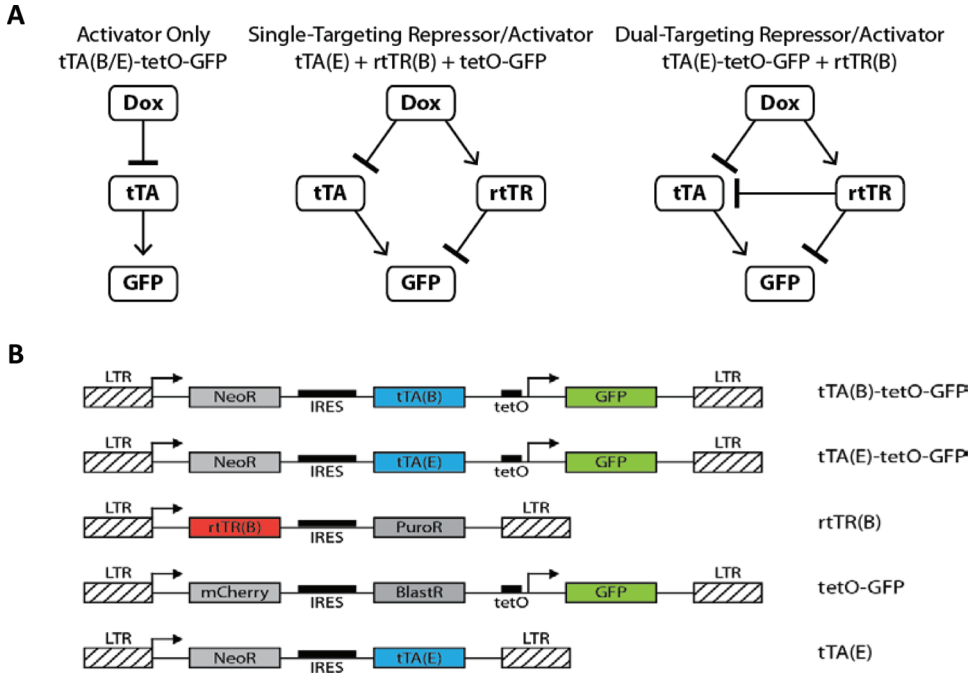

Figure 1. Summary of tetracycline-responsive expression systems. (A) Schematic diagrams for the regulation. (B) Vector maps. Doxycycline (Dox) was used instead of tetracycline in this study; "tetracycline" was used throughout in terminology as is commonly practiced. Notation: retroviral long terminal repeats (LTR), neomycin resistance gene (NeoR), puromycin resistance gene (PuroR), blasticidin resistance gene (BlastR), internal ribosome entry site (IRES), mCherry fluorescent protein (mCherry), class B tetracycline-responsive transcriptional activator (tTA(B)), class E tetracycline-responsive transcriptional activator (tTA(E)), reverse tetracycline-responsive transcriptional repressor (rtTR(B)), and tet operators (tetO). The gene of interest for all experiments was green fluorescent protein (GFP).

the targeted gene of interest. To avoid this, one must utilize orthogonal dimerization domains to ensure formation of only homodimers. Fortunately, orthogonal classes of tetR proteins have been characterized,[19] and researchers have previously demonstrated successful coexpression and activity of repressors and activators containing orthogonal tetR domains.[20,21] This study utilizes class B and class E tetR proteins, which originally were discovered encoded on transposon Tn*10* and plasmid pSL1456, respectively. These two classes share 50% amino acid sequence identify and do not readily form heterodimers with each other.[22]

We have aimed to further reduce background expression and improve the fold-expression range (i.e., "on" expression divided by "off" expression) of a tetracycline-regulated expression system. Taking advantage of the long-range repression achieved by KRAB-mediated repressors, our strategy was to use a tetracycline-responsive, orthogonally compatible repressor to control not only transcription of a gene of interest but also transcription of the activator gene encoded on the same vector. To investigate this strategy we (1) devised a computational model, (2) engineered a class E tetracycline-responsive transcriptional activator compatible with a class B tetracycline repressor, and (3) evaluated the system in cells.

Design and Strategy. Our goal was to improve the controlled expression range of a gene of interest (GOI, which in all of our experiments was green fluorescent protein (GFP)) using two synthetic transcriptional regulators: the tetracycline-responsive transcriptional activator (tTA, which releases from tetO DNA in the presence of doxycycline) and a tetracycline-responsive transcriptional repressor. The repressor employed by our system, the reverse tetracycline-responsive transcriptional repressor (rtTR), is a fusion between the KRAB repression domain and the tetR mutant domain from the rtTA ("tet-on" activator) that binds the tetO in the presence of doxycycline (Supporting Figure 1). When tTA and rtTR are coexpressed, addition of doxycycline activates binding of the rtTR and deactivates binding of the tTA activator, thus turning expression "off". Removal of doxycycline activates binding of the tTA activator and deactivates binding of the rtTR repressor, thus turning expression "on".

In previous studies, a tetracycline-responsive transcriptional activator, repressor, and a regulated gene of interest have been expressed from three independent vectors[14,15] or two vectors with activator and repressor combined on a single construct.[16] We refer to this design as the single-targeting repressor/activator, since the repressor affects expression of a single gene, the GOI (Figure 1A, middle). We hypothesized that positioning the activator cassette (i.e., retroviral vector promoter plus tTA) in a vector that also contained the tetracycline-regulated GOI (i.e., tetO, minimal promoter, and GOI) would more effectively reduce background expression in the "off" state. In this construct (tTA(B/E)-tetO-GFP in Figure 1B) the retroviral promoter driving expression of the tTA activator is a relatively long distance (3.4 kb) upstream of the tetO; the minimal promoter driving the tetracycline-regulated GOI is immediately downstream (90 bp) of the tetO. Because the KRAB repression domain in the rtTR can repress transcription over long distances up- and downstream of its DNA binding location,[18] when the rtTR binds to the tetO sites, not only should it repress transcription of the GOI, but it should also repress transcription of the tTA activator. Thus, with less tTA expressed in the "off" state, leaky GOI expression due to residual tTA binding should be decreased. Because the rtTR represses two genes, the GOI and the tTA, we refer to this design as the dual-targeting repressor/activator system (Figure 1A, right).

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Model of Tetracycline Regulated Expression Systems. To evaluate our proposed design, we devised a mass-action kinetic model described by a system of ordinary differential equations (see Supporting Table 1). We compared the dual-targeting repressor/activator system, single-targeting repressor/activator system, and the activator-only system (Figure 1A). The model simulated rtTR, tTA, and GOI levels (R_{Tot}, A_{Tot}, and G_{Tot}, respectively) at different doxycycline levels.

In the activator-only and single-targeting repressor/activator system, the total amount of tTA activator (A_{Tot}) was described by

$$\frac{dA_{Tot}}{dt} = k_A - k_{Adeg}A_{Tot} \quad (1)$$

The total repressor (R_{Tot}) or rtTR levels in both repressor/activator systems were modeled by

$$\frac{dR_{Tot}}{dt} = k_R - k_{Rdeg}R_{Tot} \quad (2)$$

Regulated expression of the GOI (G) for both activator/repressor systems was described by

$$\frac{dG}{dt} = \frac{k_G(S_{ADox} + S_{A0})}{(1 + S_{RDox} + S_{R0})^{\gamma_G}} - k_{Gdeg}G \quad (3)$$

The activator-only system without any repression was described by

$$\frac{dG}{dt} = k_G(S_{ADox} + S_{A0}) - k_{Gdeg}G \quad (4)$$

Activator-dependent expression was described by a term scaled proportionally by k_G (eqs 3 and 4). To model activator-dependent expression, k_G was multiplied by a fraction of tetO sites bound by a tTA protein. To account for leaky expression mediated by the activator in a doxycycline-independent manner, these sites could be occupied not only by tTA bound to doxycycline (S_{ADox}) but also tTA unbound (S_{A0}) to doxycyline. GOI repression, reflected in the denominator of the eq 3 expression term, was inversely dependent on the fraction of tetO sites occupied by repressor (whether bound or unbound to doxycycline). Here a parameter γ_A allowed for a nonlinear relationship between repressor levels and repression.

For the dual-targeting repressor/activator system, where the repressor also repressed expression of the activator (Figure 1B), a repression term (denominator of the activator expression term) was similarly incorporated. Specifically, the activator was represented by

$$\frac{dA_{Tot}}{dt} = \frac{k_A}{(1 + \alpha S_{RDox} + \alpha S_{R0})^{\gamma_A}} - k_{Adeg}A_{Tot} \quad (5)$$

For all systems, proteins degradation was modeled with first-order kinetics. All binding interactions, regulator-doxycycline and regulator-tetO (regulator here means activator or repressor), were modeled as equilibrium relationships (Supporting Information). Because we assumed that all key regulation happens at the transcriptional level, protein and mRNA species were not separately described. The model also simplifies binding of the regulator proteins to the promoter treating the binding proteins as single molecules and not dimers. Finally, the inclusion of an α term allowed us to specify a fractional level of repression of the activator expression relative to that of the GOI. As one would expect, as α approaches zero, the dual-targeting repressor/activator system approaches the behavior of the single-targeting repressor/activator.

The three systems were simulated at steady-state conditions (Figure 2, Supporting Figure 2) over a range of doxycycline concentrations. The models demonstrated that use of an inducible repressor in conjunction with an activator could improve the range of expression through suppression of leaky expression. Results also demonstrated that even greater improvement in the expression range could occur if the repressor was also able to reduce expression of the activator. Because not all parameter values for this model were available, we considered this model to be demonstrative and suggestive rather than predictive, though additional sensitivity analysis found consistency in the resulting trends even when parameters values were perturbed ±50% (Supporting Table 2). It reinforced our notion that the dual-targeting repression/activator system with both proximal and distal repression could potentially generate an improved expression range. We hope that our model will also provide a starting point for other researchers who intend to model activators and repressors when taking into account leaky and background expression.

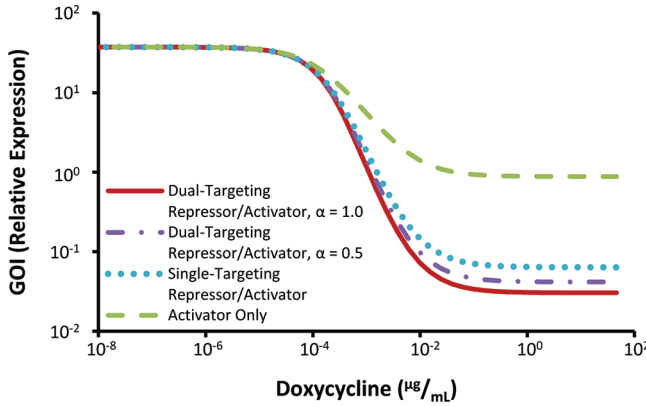

Figure 2. Model of tetracycline-responsive expression. Simulated expression of a gene of interest (GOI) using different regulation schemes and varying doxycycline concentrations. The dual-targeting repressor/activator system was modeled where the activator was either repressed with either half (α = 0.5) or all (α = 1) of the repression experienced by the GOI.

Engineering Class B and Class E tTA Activators. Following the predicted improvements in performance by the dual-targeting repressor/activator system, we proceeded to engineer it along with a single-targeting repressor/activator and activator-only system. Because rtTR and tTA genes that encode orthogonal tetR domains did not already exist, we needed to generate them. The available versions of these transcription factors used the same class B tetR domain, and in order to make a compatible pair, we replaced the class B domain with a class E domain (Figure 1B, Figure 3, Supporting Figure 1).[25,26] Because switching tetR classes could unexpectedly change the functional properties of the tTA activator, we first evaluated them alone. Through retroviral transduction, we created stable cell lines that used either a class B or class E domain in the tTA to drive GFP expression. After analysis of fluorescence intensity by flow cytometry, we found that both tTAs generated similar GFP expression ranges (approximately 9- (tTA(E)) and 13-fold (tTA(B)) in response to different concentrations of doxycycline (0 to 1 μg/mL) (Figure 4A).

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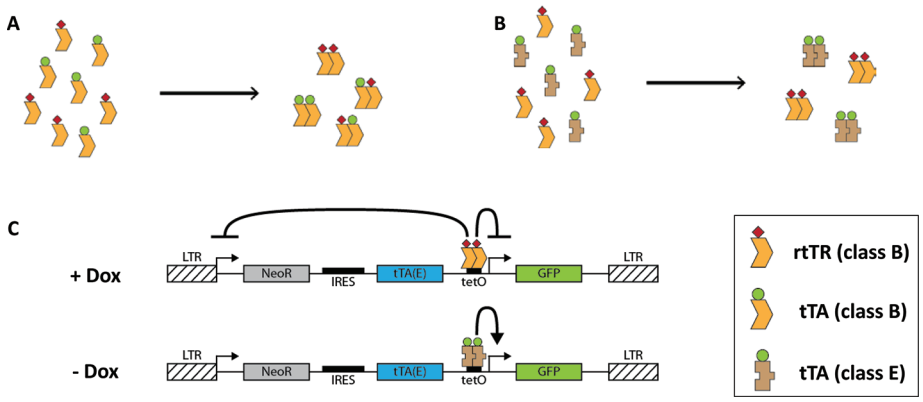

Figure 3. TetR interactions. (A) If one employs dimerization domains of the same class (e.g., class B) for repressor and activator proteins, a combination of activator homodimer, repressor homodimer, and activator/repressor heterodimer may occur. (B) In comparison, use of a class B rtTR repressor and class E tTA activator leads to only activator homodimer and repressor homodimer formation. (C) Use of class E tTA and class B rtTR in the dual targeting repressor/activator system. In the presence of doxycycline (+Dox), binding of rtTR(B) dimers to tetO and subsequent repression of both GFP and tTA expression. In the absence of doxycycline (−Dox), activation of GFP expression due to binding of tTA(E) dimers to the tetO.

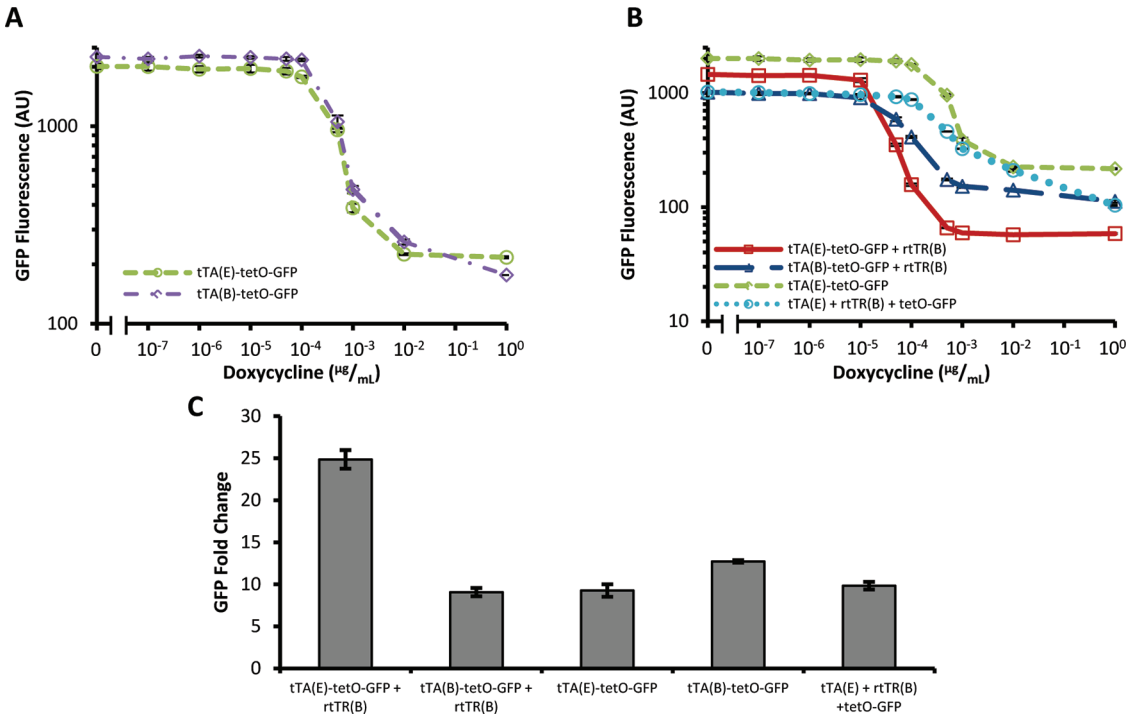

Figure 4. GFP expression regulated by addition of doxycycline. (A) GFP expression by original class B tTA (tTA(B)-tetO-GFP) and newly constructed class E tTA (tTA(E)-tetO-GFP). (B) Doxycycline-response curves of dual-targeting repressor/activator systems using different (tTA(E)-tetO-GFP + rtTR(B)) and identical (tTA(B)-tetO-GFP + rtTR(B)) dimerization domains, activator-only (tTA(E)-tetO-GFP), and single targeting repressor/activator system (tTA(E) + rtTR(B) + tetO-GFP). (C) Overall fold change in GFP between cells cultured in 1 and 0 μg/mL doxycycline.

Evaluation of the Dual-Targeting Repressor/Activator System. Having found that the newly created class E tTA had sufficient activator function, we proceeded with creating stable cell lines that expressed the class E tTA activators with class B rtTR repressors (Figure 1). Both the dual-targeting repressor/activator (tTA(E)-tetO-GFP + rtTR(B)) and single-targeting repressor/activator (tTA(E) + rtTR(B) + tetO-GFP) systems demonstrated overall fluorescence levels lower than those of the activator-only (tTA(E)-tetO-GFP) system over a range of doxycycline concentrations (Figure 4B). This suggests that in general the repressor was able to repress GFP expression. Furthermore, it not only was reducing leaky expression in the "off" state but also was contributing to a detectable level of leaky repression in the "on" states. However, using the repressor with the activator did not only shift the magnitudes of expression, since the systems also demonstrated distinct changes to their fold-expression ranges (Figure 4B,C). In this regard the dual-targeting repressor/activator was superior, demonstrating a 25-fold range in GFP expression, while the single-targeting repressor/activator and activator-only systems demonstrated only 10- and 13-fold ranges, respectively (Figure 4C). This improvement in the fold-range of expression could be attributed to ability of the dual-targeting repressor/activator to most effectively reduce leaky expression in the "off" state.

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Additionally, results suggested that the engineered class E tTA was orthogonally compatible with the class B rtTR, since the dual-targeting repressor/activator system using the orthogonal domains had had a better range of expression than the same system using the same class B domains (Figure 4B). Lastly, it was observed that the dual-targeting repressor activator (tTA(E)-tetO-GFP + rtTR(B)) produced a higher level of expression in the "on" state than the single-targeting repressor/activator (tTA(E) + rtTR(B) + tetO-GFP). This could be an artifact of selection using three versus two antibiotic resistance cassettes, respectively. It might also be due to unforeseen sequence effects unique to the vector backbones.

Improved Fold-Expression Control Achieved through Repression of Activator Transcription. To determine whether the rtTR repressor in the dual-targeting repressor/activator system repressed transcription of the tTA activator in the "off" expression state, we measured the activator mRNA levels in both the "on" and "off" states (i.e., in the absence and presence of 1 μg/mL doxycyline, respectively) (Figure 5). In the dual-targeting repressor/activator system, we found that the tTA mRNA levels decreased by 4-fold upon addition of doxycycline, indicating that the rtTR repressor was indeed reducing transcription of the tTA activator. In contrast, in the single-targeting repressor/activator system the tTA mRNA remained unchanged upon addition of doxycycline. Additionally, we found that rtTR transcription in both the dual- and single-targeting modes remained largely unchanged upon addition of doxycycline (Figure 5). This suggested that rtTR-mediated repression was not occurring in a nonspecific manner and that the rtTR specifically repressed only genes (i.e., tTA and GFP) placed in sufficient proximity to tetO sites. Taken together, our results indicated that we were able to improve doxycycline-dependent control of GFP expression by using the rtTR repressor to control transcription of not only a proximally located GFP gene but also the distally located tTA activator gene.

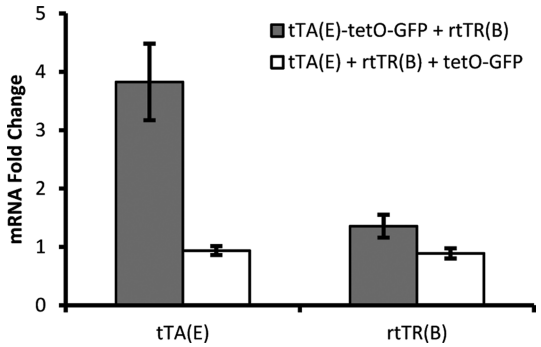

Figure 5. Expression of activator and repressor. Fold change in mRNA levels of tTA and rtTR cells when cultured in 1 and 0 μg/mL doxycycline. Dual-targeting repressor/activator (tTA(E)-tetO-GFP + rtTR(B)) and single-targeting repressor/activator (tTA(E) + rtTR(B) + tetO-GFP).

Discussion. We have engineered a tetracycline-responsive expression system with an improved, tunable expression range. This was accomplished by using a tetracycline-responsive repressor that functioned by not only directly suppressing transcription of a gene of interest but also indirectly suppressing transcription of the reciprocal tetracycline-responsive ("tet-off") activator. This dual-targeting approach effectively decreased the system's leaky expression, since the tetracycline-responsive activator still had residual activity at saturating doxycycline concentrations. Our dual-targeting repressor/activator system is also an example of a synthetic, coherent type 2 feed-forward loop (C2-FFL), since the repressor not only regulates expression of the GOI directly but also indirectly regulates the GOI by regulating the expression of the activator.[27] However, the system deviates somewhat from the canonical C2-FFL in that doxycycline not only regulates the DNA-binding of the repressor but also that of the activator. Although here we did not set out to address the dynamics of induction, we have found that in general it takes longer to turn "off" GFP expression rather than turn it "on." This could be due to the long half-life of the GFP protein and potentially slower-acting dynamics of the KRAB repressor. In the future, it would also be interesting to investigate how this particular C2-FFL motif responds dynamically.

We believe our study was novel in a few other aspects. To create the dual-targeting repressor/activator system, we needed to use tetracycline-responsive activators and repressors that would not form heterodimers. In accomplishing this, we believe that we have also created the first class E tTA activator. Additionally, while tetracycline-responsive activators and repressors have been used together before in "tet-on" systems, to our knowledge this study was the first to demonstrate the use of the activator and repressor in a "tet-off" system that operates in mammalian cells. Furthermore, while these previously combined activator and repressor systems required simultaneous use of three different vectors, our system requires only two, which for some will be an important practical advantage. We also note that we could not have created a dual-targeting repressor/activator control scheme simply by using a second tetracycline-responsive promoter to drive expression of the rtTR repressor. This would be counterproductive since in the high doxycycline "off" state, there would be less expression (due to repression) of the repressor. In the no doxycycline "on" state, there would be greater expression of the repressor, which in this study we showed still caused leaky repression at saturating doxycycline concentrations.

Tetracycline-responsive expression systems are useful not only for setting "on" and "off" states but also for specifying intermediate expression levels by supplementing intermediate levels of doxycycline. In this regard our design was apropos, since we avoided positive feedback mechanisms that could lead to bistability. Here, instead to improve the dose–response to doxycycline, we leveraged the ability of the rtTR to repress over long distances. Because the KRAB repression domain in the rtTR can also affect the chromatin structure several kilobases up and downstream of its DNA binding site, here the rtTR also worked to repress expression of an upstream activator transcribed from a promoter with no tetO sites and on its own would act as a constitutive promoter. Our work should encourage synthetic biologists to characterize and take advantage of the long- or short-range abilities of synthetic transcription factors.

METHODS

Modeling. Model equations were evaluated using Matlab version R2011b (MathWorks, Natick, MA). Numerical approximation was performed using the ode45 command, a fourth order Runge–Kutta approximation algorithm. Initial values of 1 were used for all proteins, and equations were solved out to a time of 1000 h to ensure that steady-state values were reached.

Vectors. The retroviral vectors used in this study were originally derived from the Moloney murine leukemia virus. Vectors were created using standard PCR and plasmid construction methods. The rtTR was amplified by PCR from pLVPT-rtTR-KRAB-2SM2[23] and inserted upstream of an internal ribosome entry site (IRES) and puromycin resistance gene. The tTA(E) gene was generated by removing the sequence encoding the tetR class B dimerization domain and replacing it with a tetR class E dimerization domain (Supporting Figure 1). The class E dimerization domain [GenBank: X14035] was synthesized by Integrated DNA Technologies (Coralville, IA). All tetracycline-responsive promoters consist of seven wild-type tetO binding sites (5′-TCCCTATCAGTGATAGAGA-3′) upstream of the minimal cytomegalovirus immediate-early (CMV) basal promoter element.

Cell Engineering and Culture. HEK-293 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin; PD-31 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin (FBS was from Gemini Bio-Products, Sacramento, CA; all other media and supplements were from Life Technologies, Carlsbad, CA). Cells were incubated in 5% CO_2 at 37 °C. Selection was performed by addition of 2 μg/mL puromycin, 10 μg/mL blasticidin, and/or 1 mg/mL Geneticin (Life Technologies) to the relevant PD31 cell lines. Activator, repressor, and reporter constructs were stably expressed by infection with retroviral particles, which were generated by cotransfection of the expression vectors with the ecotropic pCL-Eco packaging vector[24] in HEK-293 cells. GFP-positive cells were then sorted by fluorescence-activated cell sorting (FACS) using an Aria II (Becton Dickinson, Franklin Lakes, NJ) for GFP positive cells.

Doxycycline Expression Response Measurements. Cultures of cells containing the various activator, repressor, and reporter constructs were seeded in triplicate at a density of 3×10^4 cells/mL in media containing 0, 1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 5×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 1×10^{-2}, and 1 μg/mL doxycycline (Fisher Bioreagents, Fair Lawn, NJ). The media was additionally supplemented with 1 μg/mL puromycin and 1 mg/mL Geneticin for all cell lines with 5 μg/mL blasticidin additionally added to the 3-vector (tTA(E) + rtTR(B) + tetO-GFP, see Figure 1A for vector maps) cell line. Following 3 days of incubation, the cells were analyzed for GFP fluorescence by flow cytometry (FACScan, Becton Dickinson). All fluorescence values were adjusted for autofluorescence, which was determined by analyzing PD31 cells lacking any GFP expression construct.

Quantification of mRNA Levels. PD-31 cells containing either single-targeting repressor or double-acting repressor expression systems were cultured in triplicate in 0 and 1 μg/mL doxycycline for 3 days. Total RNA was then extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA) and analyzed by quantitative real-time PCR on a StepOnePlus PCR Machine (Applied Biosystems). Quantification was performed using primers that recognized sequences from the vp16 domain of the tTA or the KRAB domain of the rtTR (see Supporting Table 3 for primer sequences). All reactions were performed in duplex with VIC-MGD labeled murine GAPDH primers (Applied Biosystems, Product no. 4308313) as an mRNA reference and FAM-labeled ZEN double quenched primers (Integrated DNA Technologies, Coralville, IA) for the experimental genes (VP16, KRAB).

■ ASSOCIATED CONTENT

Supporting Information

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Notes

The authors declare no competing financial interest.

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