Regulation of Endogenous Human Gene Expression by Ligand-Inducible TALE Transcription Factors

Andrew C. Mercer,[†] Thomas Gaj,[†] Shannon J. Sirk, Brian M. Lamb, and Carlos F. Barbas, III*

The Skaggs Institute for Chemical Biology and the Departments of Chemistry and Cell and Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, United States

S Supporting Information

ABSTRACT: The construction of increasingly sophisticated synthetic biological circuits is dependent on the development of extensible tools capable of providing specific control of gene expression in eukaryotic cells. Here, we describe a new class of synthetic transcription factors that activate gene expression in response to extracellular chemical stimuli. These inducible activators consist of customizable transcription activator-like effector (TALE) proteins combined with steroid hormone receptor ligand-binding domains. We demonstrate that these ligand-responsive TALE transcription factors allow for tunable and conditional control of gene activation and can be used to regulate the expression of endogenous genes in human cells. Since TALEs can be designed to recognize any contiguous DNA sequence, the conditional gene regulatory system described herein will enable the design of advanced synthetic gene networks.

KEYWORDS: TALE, inducible gene expression, transcription factor, steroid hormone receptor, gene regulation
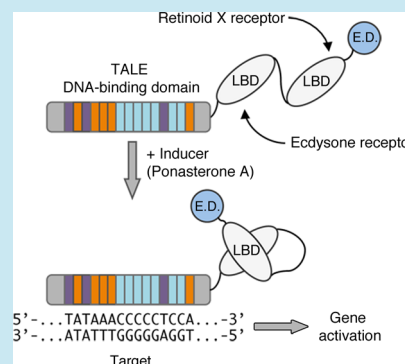
Gene regulatory networks are complex biological circuits that control a wide variety of cell-type specific phenotypes. One aim of synthetic biology is to recapitulate this level of regulation within synthetic biological circuits[1] to enable a range of biotechnological[2] and therapeutic applications.[3] To date, prokaryotic regulatory systems (e.g., *lac, tet,* and *ara*)[4] have proven tremendously valuable for the construction and implementation of synthetic genetic circuitries. Such examples include toggle switching,[5] counting,[6] biocomputation,[7−9] timing,[10,11] oscillation,[12−15] communication,[16] and controlled expression of therapeutic genes.[17] Many of these circuits, however, are based on a limited set of simple modular components. Although these elements allow for straightforward and rapid assembly of synthetic devices, they may not be optimal for the design of sophisticated transcriptional networks[18] that rely on endogenous genetic elements or coregulatory factors. As such, there is a pressing need for adaptable tools capable of providing highly regulated control of gene expression in eukaryotic cells.

Transcription activator-like effectors (TALEs) are a class of naturally occurring DNA-binding proteins that can be utilized for the design of hybrid DNA-modifying enzymes.[19] A typical TALE DNA-binding domain consists of a series of 33- to 35- amino acid repeats that each recognizes a single base pair (bp) of DNA via two adjacent amino acid residues, termed repeat variable diresidues (RVDs) (Figure 1).[20,21] TALE repeats can be assembled into custom DNA-binding domains capable of recognizing virtually any contiguous DNA sequence. Synthetic TALE arrays have been fused to numerous effector domains including transcriptional activators[22,23] and repressors,[24] nucleases,[22,25,26] and site-specific recombinases.[27] Because of

this versatility and the relative ease with which they can be constructed, TALEs have found broad utility in biotechnology[28] and the biomedical sciences.[29,30]

Recent studies have demonstrated the effectiveness of TALEs for synthetic biology.[31−33] Most notably, combinations of TALE transcription factors (TALE-TFs) have been shown to synergistically activate gene expression in human cells,[34,35] providing synthetic biologists with a framework for devising novel circuits that mimic the complexity of natural systems. Unlike the more commonly used tetracycline-inducible system[36] used in the first study to successfully impart chemical control over endogenous gene expression with designed transcription factors,[37] TALE-TFs can be engineered as two-hybrid systems to allow for gene induction only in the presence of light[38] or endogenous stimuli.[39] These approaches, however, are limited by numerous factors, including low inducer sensitivity, toxicity, and the necessity dual-gene systems to accommodate the two-hybrid approach. To address this, we set out to develop a versatile new class of ligand-dependent transcriptional activators by combining steroid hormone receptor ligand-binding domains (LBDs) with custom-designed TALE-TFs, a strategy that permits conditional activation of gene expression in response to small-molecule stimuli. Such an approach should allow for the control of a variety of TALE-TFs using a number of distinct small molecules, and facilitate the
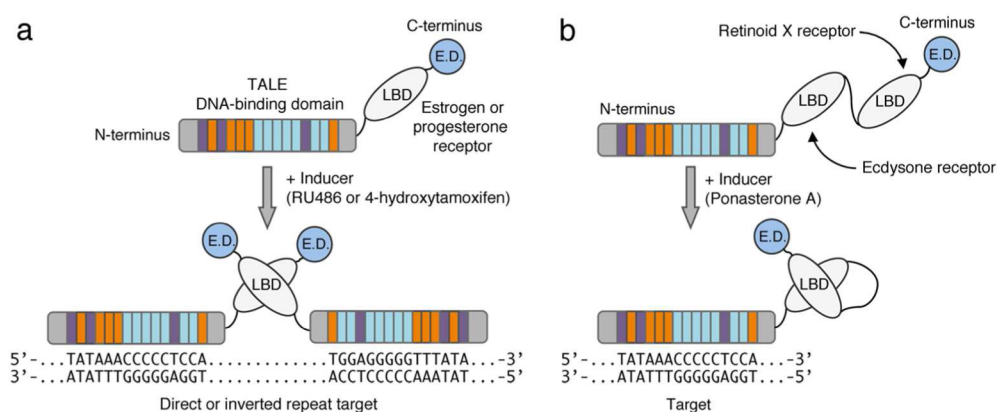
Figure 1. Ligand-inducible TALE transcription factors. (a) TALE-TF proteins fused to ligand-binding domains (LBDs) from the estrogen receptor (ER) or progesterone receptor (PR) undergo intermolecular dimerization in response to 4-hydroxytamoxifen (4-OHT) or RU486, respectively, and up-regulate gene activation from DNA sequences that contain two direct or inverted repeat TALE binding sites. (b) TALE-TF proteins fused to the chimeric single-chain retinoid X-α/ecdysone (RXE) LBD undergo intramolecular rearrangement in response to ponasterone A (PonA) and up-regulate gene activation from target DNA that contains only a single TALE binding site. E.D. indicates effector domain.

design of highly responsive synthetic networks that interact with endogenous genetic components.

We began by fusing the LBDs from the murine estrogen (ER) and human progesterone (PR) receptors to a synthetic 15-repeat TALE protein designed to recognize the AvrXa7 binding site linked to the VP64[40] transactivation domain (Avr15-VP64). The ER and PR proteins are prototypical members of the nuclear steroid hormone receptor family. In the absence of hormone, these receptors are inactive due to association with several destabilizing factors. Upon ligand binding, however, these hormone receptors are released by the inactivating complex, undergo intermolecular homodimerization, and bind their target DNA to activate transcription (Figure 1a). We[41−43] and others[44,45] have previously shown that zinc-finger transcription factors[46] fused to these LBDs modulate gene expression in response to specific chemical stimuli. As the natural ligands of these mammalian receptors mediate significant biological activity and thus may stimulate induction of unwanted and potentially confounding gene networks, we employed mutants of the ER[47] and PR[48] proteins that no longer bind their natural substrates but instead recognize the synthetic agonists 4-hydroxytamoxifen (4-OHT) and RU486, respectively. We also investigated whether Avr15-VP64 could activate gene expression in response to the insect hormone ponasterone A (PonA) when fused to a single-chain regulator composed of LBDs from the retinoid X-α/ecdysone (RXE) heterodimeric receptor systems (Figure 1b).[41,42,49] Unlike the ER and PR proteins, which must dimerize to become active, the single-chain RXE protein functions as a monomer, as RXEs undergo an intramolecular rearrangement in response to ligand, enabling gene regulation from a single DNA binding site. All LBDs were fused between the C-terminus of the TALE DNA-binding domain and the N-terminus of the VP64 transactivation domain, as illustrated in Figure 1.

To assess the ligand-responsiveness of each chimeric TF, we developed a transient reporter assay that correlates gene activation with increased luciferase expression. We introduced five direct repeats of the 15-bp Avr15 TALE binding site upstream of a luciferase reporter gene and cotransfected human embryonic kidney (HEK) 293T cells with reporter plasmid and expression vectors for each TALE-TF (Figure 2a). Luciferase expression was measured 24 h after introduction of 4-OHT,
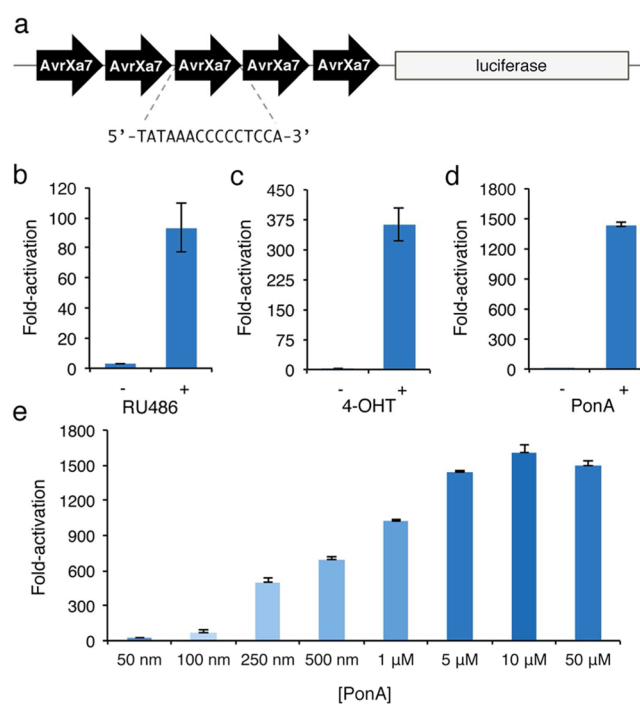

Figure 2. Activation of gene expression by ligand-responsive TALE transcription factors. (a) Schematic illustration of the luciferase reporter system used to evaluate ligand-inducible TALE-TF gene activation. Avr15 target site repeats indicated. (b−d) Fold-activation of luciferase expression observed with the (b) PR/RU486, (c) ER/4-OHT, and (d) RXE/PonA regulatory systems. (e) Fold activation of luciferase expression observed by RXE-TFs with increasing concentrations of PonA. Error bars indicate standard deviation ($n = 3$; p-value < 0.0005; paired t-test).

RU486, or PonA. In the absence of ligand, we observed negligible amounts of luciferase expression; however, in the presence of the appropriate small-molecule, we observed robust gene expression for each Avr15-VP64 fusion (Figure 2b−d). Notably, each ligand-inducible TF stimulated gene activation only in the presence of its intended inducer (Supporting Information Figure S1). This orthogonality suggests that these systems could be used for the construction of multiplexed gene regulatory networks. Notably, the single-chain RXE-Avr15-
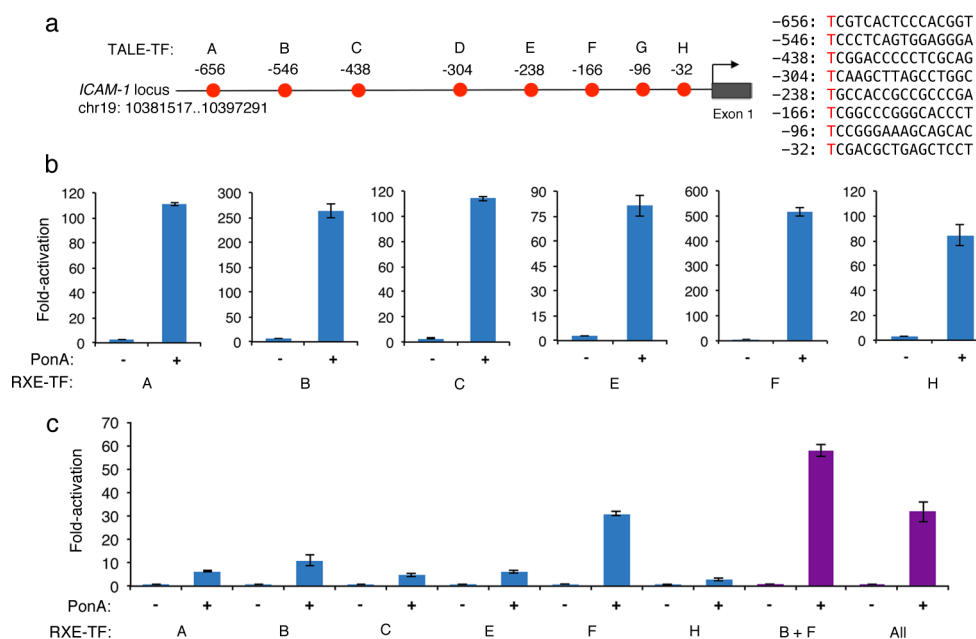
Figure 3. Ligand-dependent gene activation from the *ICAM-1* promoter by RXE-TFs. (a) (left) Location (red circles) of the TALE binding sites within the ICAM-1 promoter region, and (right) the sequence of each target site. 5′ thymidine nucleotides highlighted red. (b and c) Fold activation of luciferase expression after co-transfection of RXE-TF expression plasmids with luciferase reporter plasmids containing (b) four direct repeats of the *ICAM-1* TF binding site or (c) the full-length *ICAM-1* promoter upstream of a luciferase reporter gene. Error bars indicate standard deviation (n = 3; p-value < 0.0005; paired t-test).

VP64 activator demonstrated substantially higher levels of gene activation (∼1400-fold) than the homodimeric PR-Avr15-VP64 (∼90-fold) and ER-Avr15-VP64 (∼350-fold) proteins after induction. All subsequent experiments were, therefore, performed using the RXE regulatory system.

To determine whether the RXE LBD regulates gene expression over a wide range of ligand concentrations, we evaluated the dependence of gene activation by the RXE-Avr15-VP64 protein on PonA concentration. We found that luciferase expression increased linearly in response to PonA concentrations from 100 nM to 5 μM and that the highest level of induction occurred with 10 μM PonA (>1600-fold) (Figure 2e). We observed no luciferase expression with PonA concentrations less than 50 nM. Intriguingly, the ER and PR proteins exhibited switch-like behavior by inducing high levels of gene expression in the presence of 10 nM 4-OHT and 1 nM RU486, respectively (Supporting Information Figure S2), indicating that the ER and PR domains are less tunable than the RXE LBD. Taken together, these findings indicate that RXE-based TFs are sufficiently sensitive for applications that require tunable forms of gene activation.

Next, we sought to determine whether the RXE-based activators could regulate gene expression at target sites derived from human promoter regions. To test this possibility, we constructed eight RXE-TFs that target distinct sites located within 700-bp of the transcriptional start site (TSS) of the *intercellular adhesion molecule 1* (*ICAM-1*) gene. Abnormal ICAM-1 levels are associated with inflammation and athero-sclerosis. All eight TALEs were composed of 14.5 repeats (in order to target 15-bp sequences) and designed to target sequences across the entire promoter region in the 5′ to 3′ orientation (Figure 3a). Notably, TALE design was constrained only by the standard requirement for a 5′ thymidine nucleotide. In the future, this restriction might be removed as a recent report has described the development of TALE frameworks

that demonstrate no base restrictions at this position and allow for any sequence to be targeted.[50] To assess the potency of each individual RXE-TF, we created luciferase reporter constructs containing four direct repeats of each individual TALE target upstream of the luciferase gene (Figure 3a). We cotransfected HEK293T cells with a single reporter plasmid and the corresponding RXE-TF expression vector and measured luciferase expression 24 h after induction with 5 μM PonA. Six of the eight RXE-TFs (A, B, C, E, F, and H) increased luciferase expression by more than 75-fold in response to PonA, and negligible amounts of gene expression occurred in the absence of hormone (Figure 3b). The most potent activator, RXE-TF F, enhanced luciferase expression by more than 500-fold after induction with hormone (Figure 3b). Interestingly, we found that two of the eight RXE-TFs (D and G) were unable to induce significant levels of gene expression in response to PonA. Further analysis revealed that the corresponding constitutively expressed (noninducible) TALE-TFs were also unable to significantly enhance gene expression, suggesting an inherent limitation within these TALEs or their target sites (Supporting Information Figure S3). In general, the RXE-TFs enhanced gene expression to within 2- to 5-fold that of constitutively expressed TFs (Supporting Information Figure S3).

Next, we evaluated whether the active RXE-TFs could regulate gene expression from a full-length promoter sequence. To test this, we cotransfected HEK293T cells with a reporter plasmid that contained ∼1000 bp of the *ICAM-1* promoter with expression vectors for each individual *ICAM-1* RXE-TF. With the exception of RXE-TF F, which activated luciferase expression ∼30-fold, we found that each RXE-TF (A, B, C, E, and H) induced only modest levels of gene activation (Figure 3c). However, we found that cotransfection and simultaneous induction with combinations[34,35] of RXE-TFs led to an apparent synergistic increase in gene activation. In
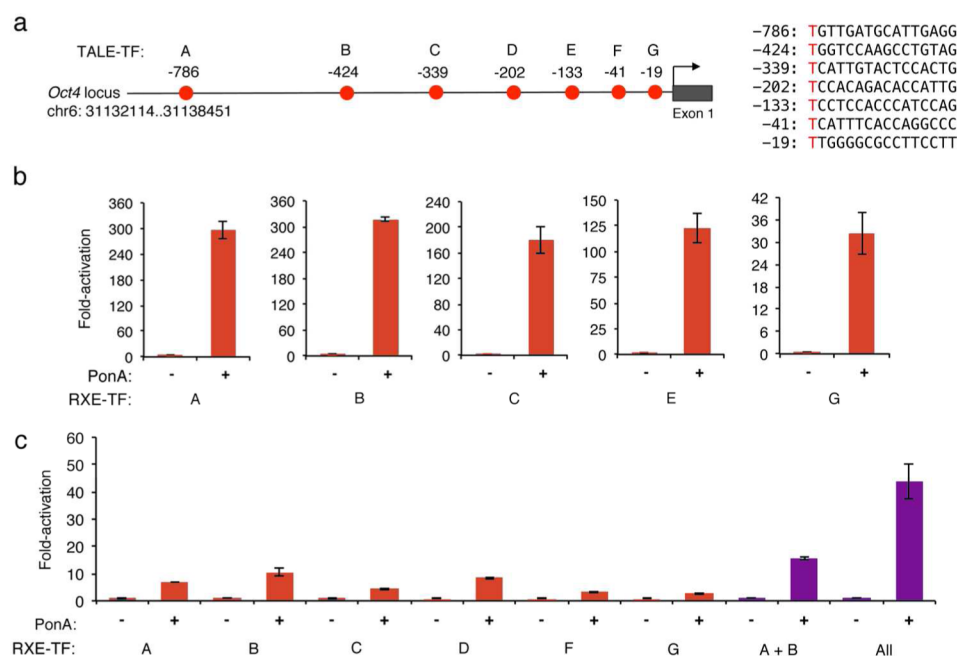
Figure 4. Ligand-dependent gene activation from the *Oct-4* promoter by RXE-TFs. (a) (Left) Location (red circles) of the TALE binding sites within the *Oct-4* promoter region, and (right) the sequence composition of each target site. 5′ thymidine nucleotides highlighted red. (b and c) Fold activation of luciferase expression after co-transfection of RXE-TF expression plasmids with luciferase reporter plasmids containing (b) four direct repeats of the *Oct-4* TF binding site or (c) the full-length *Oct-4* promoter upstream of a luciferase reporter gene. Error bars indicate standard deviation ($n = 3$; p-value < 0.0005; paired t-test).
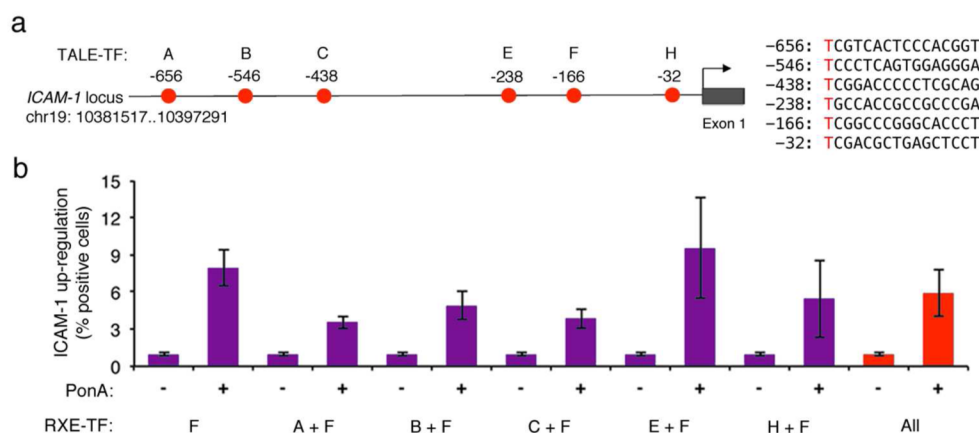

Figure 5. Regulation of endogenous ICAM-1 expression by RXE-TFs. (a) Location (red circles) of the TALE-binding sites within the *ICAM-1* promoter region, and (right) the sequence composition of each target site. (b) Percentage of HeLa cells that showed an increase in ICAM-1 expression by flow cytometry after transfection with RXE-TFs targeting the endogenous ICAM-1 promoter. Data was normalized to mock-transfected cells. Error bars indicate standard deviation ($n = 3$; p-value < 0.002; paired t-test).

particular, codelivery of RXE-TFs B and F led to a ∼65-fold increase in luciferase expression in the presence of PonA (Figure 3c). Intriguingly, simultaneous induction of all six RXE-TFs led to only a ∼30-fold increase in gene activation, indicating that the relative positions of the TALE binding sites impact cooperative gene activation.

To examine the generality of the RXE regulatory system, we constructed and tested seven RXE-TFs composed of 14.5 repeats and designed to target sites within 800 bp of the TSS of the human *octamer-binding transcription factor 4* (*Oct-4*) gene, which has been implicated in both tumorigenesis and pluripotency (Figure 4a). Five of the seven RXE-TFs (A, B, C, E, and G) induced luciferase expression at levels near that of constitutively expressed TFs (Figure 4b and Supporting

Information Figure S4). In particular, two RXE-TFs (A and B) increased luciferase expression by more than 300-fold in response to PonA. We note that four direct repeats of the binding sites were also used in these reporter systems. To evaluate whether these RXE-TFs could also regulate gene expression in the context of the full promoter sequence with a single TF binding site, we cotransfected a luciferase reporter plasmid that harbored ∼1000 bp of the *Oct-4* promoter with individual RXE-TFs. As with TFs targeting the *ICAM-1* promoter, individual *Oct-4*-targeting RXE-TFs demonstrated only modest levels of gene activation, but cotransfection of combinations of these TFs led to ∼40-fold increases in luciferase expression in the presence of hormone (Figure 4c).

Lastly, we sought to determine whether RXE-TFs could regulate the expression of an endogenous human gene. To investigate this possibility, we transfected HeLa cells with combinations of the most potent RXE-TFs designed to target the *ICAM-1* promoter and then evaluated surface expression of ICAM-1 via flow cytometry (Figure 5a and Supporting Information Figure S5). In the absence of hormone, we detected no appreciable increase in ICAM-1 expression relative to expression on mock-transfected cells for any combination of RXE-TFs. At 48 h after induction with 10 μM PonA, however, we observed that ~5−10% of all cells transfected with individual (F) and multiple combinations of RXE-TFs (B/F; E/F; H/F; and all RXE-TFs) showed an increase in ICAM-1 expression (Figure 5b). In particular, ~10% of HeLa cells cotransfected with RXE-TF F or with the combination of RXE-TFs E and F showed a significant increase in ICAM-1 expression. Most notably, we found that the RXE-TFs upregulated ICAM-1 expression at rates within 2-to 3-fold the corresponding constitutively expressed TFs (Supporting Information Figure S6). These values are similar to those previously reported with ligand-inducible zinc-finger transcription factors;[42] however, alternate delivery methods[42,51,52] could be used to improve the potency of the RXE-TFs. Finally, because the *Oct-4* locus is likely epigenetically silenced in HeLa cells,[53] we could not assess the ability of the *Oct-4*-targeting RXE-TFs to induce activation of the endogenous gene in this particular cell type. Taken together, these findings suggest that, when coupled with TALE technology, the RXE regulatory system is a generally effective means for regulating the expression of endogenous human genes.

Fusion of designed TALE-TFs with LBDs derived from steroid hormone receptors enabled the generation of a new class of potent, adaptable, and conditional transcriptional activators. Our most active transcriptional regulator was controlled by the retinoid X-α/ecdysone receptor system and effectively regulated transgene expression in response to the insect hormone ponasterone A in a dose-dependent manner. These ligand-responsive activators also regulated expression of an endogenous human gene at levels that approached those observed with constitutively expressed transcriptional activators. Unlike recently described light-sensitive zinc-finger[54] and TALE[38] transcriptional activators, this hormone-responsive gene regulatory system is not based on a two-hybrid system (i.e., single-gene system) and thus may be simpler to implement within gene networks. Synthetic sensors and circuits have been constructed using a well-defined suite of bacterial TF-promoter systems, and the use of artificial zinc-finger transcription factors has been shown to be an effective means for wiring synthetic transcriptional circuits in eukaryotic cells.[55] The range of sequences that can be rapidly targeted to novel sequences by these components is limited, however. The stringent, dose-responsive, and conditional gene regulatory system described herein will provide synthetic biologists with a new means for wiring and rewiring similar types of circuits with the added potential of multiplexing.

An important goal for future studies will be to determine the extent of off-target effects induced by the RXE-TFs, as recent studies have indicated that TALEs composed of 18.5 repeats can potentially tolerate up to 3 nucleotide mismatches.[56] High-throughput RNA sequencing (RNA-seq) could be used to evaluate off-target transcriptional activation.[57] Finally, although it remains unknown whether recently described CRISPR/Cas-based transcriptional modulators[57−63] are compatible with the

regulatory systems described in this work, this active area of research could dramatically expand the utility of this approach.

■ METHODS

Plasmid Construction. TALE arrays were constructed as previously described[26] using the Golden Gate TALE assembly protocol described by Cermak et al.[64] Synthesized TALE arrays were released by SfiI digestion and ligated into the same restriction sites of the previously described expression vectors pcD-ER-E2C-VP64,[41] pcD-PR-E2C-VP64,[41] pcD-RXE-E2C-VP64,[41] and pcD-E2C-VP64[40] to generate pcD-ER-Avr15-VP64, pcD-PR-Avr15-VP64, pcD-RXE-Avr15-VP64, pcD-RXE-ICAM-1−1, , 3...8-VP64, pcD-ICAM-1−1,2,3...8-VP64, pcD-RXE-Oct-4−1,2,3...7-VP64, and pcD-Oct-4−1,2,3...7-VP64. Correct assembly of each TALE construct was verified by sequence analysis (Supporting Information Table S1).

To generate luciferase reporter plasmids, a fragment of the *luciferase* gene was PCR amplified with the primers 5′ TALE-Luc-AvrXa15, 5′ TALE-Luc-ICAM-1 1,2,3...8, or 5′ TALE-Luc-Oct-4 1,2,3...7 and 3′ Luc-Rev. PCR products were digested with XhoI and SphI and ligated into the same restriction sites of pGL3-Basic (Promega) to generate pGL3-ICAM-1 1,2,3...8 and pGL3-Oct-4 1,2,3...7. The *Oct-4* promoter was PCR amplified from genomic DNA isolated from HEK293 cells with the primers 5′ Oct-4-Prm and 3′ Oct-4-Rev. The PCR product was digested with SacI and *Hin*dIII and ligated into pGL3 Basic to generate pGL3-Oct-4. The pGL3-ICAM-1 reporter plasmid has been previously described.[41] Correct construction of each plasmid was verified by sequence analysis. All primer sequences are provided in Supporting Information Table S2.

Luciferase Assays. HEK293T cells (American Type Culture Collection; ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) Antibiotic-Antimycotic (Gibco). Cells were seeded onto 96-well plates at a density of 4×10^4 cells per well and established in a humidified 5% CO_2 atmosphere at 37 °C. At 24 h after seeding, cells were transfected with 200 ng (total) of TALE-TF expression vectors (individual or combinations), 5 ng of pGL3 reporter plasmid, and 1 ng of pRL-CMV (Promega) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. At 24 h after transfection, cells were incubated with 50 nM to 50 μM PonA (Life Technologies), 10 nM RU486 (Enzo Life Sciences), or 100 nM 4-OHT (Sigma-Aldrich). At 24 h after induction, cells were lysed with Passive Lysis Buffer (Promega), and luciferase expression was determined with the Dual-Luciferase Reporter Assay System (Promega) using a Veritas Microplate Luminometer (Turner Biosystems). Data are expressed as fold-activation, which was calculated as the average luciferase activity of triplicate samples normalized to the luciferase activity of cells transfected with pGL3 reporter plasmid and an empty expression vector. *Renilla* luciferase expression from pRL-CMV was used to normalize for transfection efficiency and cell number.

Flow Cytometry. HeLa cells (ATCC) were maintained in DMEM containing 10% FBS and 1% antibiotic-antimycotic. Cells were seeded onto 24-well plates at a density of 1×10^5 cells per well and established in a humidified 5% CO_2 atmosphere at 37 °C. At 24 h after seeding, cells were transfected with 800 ng (total) of TALE-TF (individual or combinations) expression vectors using Lipofectamine 2000, according to the manufacturer's instructions. At 24 h after

transfection, cells were incubated with 10 μM PonA. At 48 h after induction, cells were harvested and incubated on ice for 30 to 60 min with PE-conjugated mouse anti-human CD54 (BD Biosciences). Cells were washed and resuspended with PBS/1% FBS, and cell-surface expression of ICAM-1 was measured by flow cytometry (FACScan Dual Laser Cytometer; BD Biosciences; FACSDiva software). For each sample, 10 000 live events were collected, and data were analyzed using FlowJo (Tree Star, Inc.). Relative ICAM-1 induction is expressed as the increase in the percentage of PE-positive cells in comparison to mock-transfected cells.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Analysis of ER, PR, and RXE orthogonality; dose−response assays for ER and PR proteins; luciferase assays of constitutively expressed TALE-TFs targeting the *ICAM-1* and *Oct-4* promoter regions; flow cytometry analysis of HeLa cells transfected with constitutively expressed TALE-TFs targeting the endogenous *ICAM-1* promoter; representative histograms for flow cytometry analysis; amino acid sequences of the TALE constructs used in this study; sequences of the primers used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: carlos@scripps.edu.
Author Contributions
[†]A.C.M. and T.G. contributed equally to this work. A.C.M. and C.F.B. conceived of the project; A.C.M., T.G., and C.F.B. designed experiments; A.C.M and B.M.L. constructed TALEs; T.G. constructed reporter vectors; A.C.M, T.G., and S.J.S performed experiments; A.C.M., T.G., S.J.S., and C.F.B. analyzed data; A.C.M, T.G., S.J.S., and C.F.B wrote the manuscript.
Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS

TALE, transcription activator-like effector; LBD, ligand-binding domain; ER, estrogen receptor; PR, progesterone receptor; RXE, retinoid X-α/ecdysone receptor; 4-OHT, 4-hydroxyta-moxifen; PonA, ponasterone A; bp, base pair

■ REFERENCES

(1) Slusarczyk, A. L., Lin, A., and Weiss, R. (2012) Foundations for the design and implementation of synthetic genetic circuits. *Nat. Rev. Genet.* 13, 406−420.

(2) Khalil, A. S., and Collins, J. J. (2010) Synthetic biology: Applications come of age. *Nat. Rev. Genet.* 11, 367−379.

(3) Folcher, M., and Fussenegger, M. (2012) Synthetic biology advancing clinical applications. *Curr. Opin. Chem. Biol.* 16, 345−354.

(4) Auslander, S., and Fussenegger, M. (2013) From gene switches to mammalian designer cells: present and future prospects. *Trends Biotechnol.* 31, 155−168.

(5) Gardner, T. S., Cantor, C. R., and Collins, J. J. (2000) Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 403, 339−342.

(6) Friedland, A. E., Lu, T. K., Wang, X., Shi, D., Church, G., and Collins, J. J. (2009) Synthetic gene networks that count. *Science* 324, 1199−1202.

(7) Auslander, S., Auslander, D., Muller, M., Wieland, M., and Fussenegger, M. (2012) Programmable single-cell mammalian biocomputers. *Nature* 487, 123−127.

(8) Regot, S., Macia, J., Conde, N., Furukawa, K., Kjellen, J., Peeters, T., Hohmann, S., de Nadal, E., Posas, F., and Sole, R. (2011) Distributed biological computation with multicellular engineered networks. *Nature* 469, 207−211.

(9) Tamsir, A., Tabor, J. J., and Voigt, C. A. (2011) Robust multicellular computing using genetically encoded NOR gates and chemical 'wires'. *Nature* 469, 212−215.

(10) Danino, T., Mondragon-Palomino, O., Tsimring, L., and Hasty, J. (2010) A synchronized quorum of genetic clocks. *Nature* 463, 326−330.

(11) Imanishi, M., Yamamoto, K., Yamada, H., Hirose, Y., Okamura, H., and Futaki, S. (2012) Construction of a rhythm transfer system that mimics the cellular clock. *ACS Chem. Biol.* 7, 1817−1821.

(12) Elowitz, M. B., and Leibler, S. (2000) A synthetic oscillatory network of transcriptional regulators. *Nature* 403, 335−338.

(13) Atkinson, M. R., Savageau, M. A., Myers, J. T., and Ninfa, A. J. (2003) Development of genetic circuitry exhibiting toggle switch or oscillatory behavior in *Escherichia coli*. *Cell* 113, 597−607.

(14) Fung, E., Wong, W. W., Suen, J. K., Bulter, T., Lee, S. G., and Liao, J. C. (2005) A synthetic gene-metabolic oscillator. *Nature* 435, 118−122.

(15) Stricker, J., Cookson, S., Bennett, M. R., Mather, W. H., Tsimring, L. S., and Hasty, J. (2008) A fast, robust and tunable synthetic gene oscillator. *Nature* 456, 516−519.

(16) Bacchus, W., Lang, M., El-Baba, M. D., Weber, W., Stelling, J., and Fussenegger, M. (2012) Synthetic two-way communication between mammalian cells. *Nat. Biotechnol.* 30, 991−996.

(17) Gitzinger, M., Kemmer, C., El-Baba, M. D., Weber, W., and Fussenegger, M. (2009) Controlling transgene expression in subcutaneous implants using a skin lotion containing the apple metabolite phloretin. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10638−10643.

(18) Lu, T. K., Khalil, A. S., and Collins, J. J. (2009) Next-generation synthetic gene networks. *Nat. Biotechnol.* 27, 1139−1150.

(19) Mak, A. N., Bradley, P., Bogdanove, A. J., and Stoddard, B. L. (2013) TAL effectors: Function, structure, engineering, and applications. *Curr. Opin. Struct. Biol.* 23, 93−99.

(20) Moscou, M. J., and Bogdanove, A. J. (2009) A simple cipher governs DNA recognition by TAL effectors. *Science* 326, 1501.

(21) Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., and Bonas, U. (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326, 1509−1512.

(22) Miller, J. C., Tan, S., Qiao, G., Barlow, K. A., Wang, J., Xia, D. F., Meng, X., Paschon, D. E., Leung, E., Hinkley, S. J., Dulay, G. P., Hua, K. L., Ankoudinova, I, Cost, G. J., Urnov, F. D., Zhang, H. S., Holmes, M. C., Zhang, L., Gregory, P. D., and Rebar, E. J. (2011) A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* 29, 143−148.

(23) Sander, J. D., Dahlborg, E. J., Goodwin, M. J., Cade, L., Zhang, F., Cifuentes, D., Curtin, S. J., Blackburn, J. S., Thibodeau-Beganny, S., Qi, Y., Pierick, C. J., Hoffman, E., Maeder, M. L., Khayter, C., Reyon, D., Dobbs, D., Langenau, D. M., Stupar, R. M., Giraldez, A. J., Voytas, D. F., Peterson, R. T., Yeh, J. R., and Joung, J. K. (2011) Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat. Methods* 8, 67−69.

(24) Cong, L., Zhou, R., Kuo, Y. C., Cunniff, M., and Zhang, F. (2012) Comprehensive interrogation of natural TALE DNA-binding modules and transcriptional repressor domains. *Nat. Commun.* 3, 968.

(25) Reyon, D., Tsai, S. Q., Khayter, C., Foden, J. A., Sander, J. D., and Joung, J. K. (2012) FLASH assembly of TALENs for high-throughput genome editing. *Nat. Biotechnol. 30*, 460−465.

(26) Mussolino, C., Morbitzer, R., Lutge, F., Dannemann, N., Lahaye, T., and Cathomen, T. (2011) A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res. 39*, 9283−9293.

(27) Mercer, A. C., Gaj, T., Fuller, R. P., and Barbas, C. F., 3rd. (2012) Chimeric TALE recombinases with programmable DNA sequence specificity. *Nucleic Acids Res. 40*, 11163−11172.

(28) Gaj, T., Gersbach, C. A., and Barbas, C. F., 3rd. (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol. 31*, 397−405.

(29) Ousterout, D. G., Perez-Pinera, P., Thakore, P. I., Kabadi, A. M., Brown, M. T., Qin, X., Fedrigo, O., Mouly, V., Tremblay, J. P., and Gersbach, C. A. (2013) Reading frame correction by targeted genome editing restores dystrophin expression in cells from Duchenne muscular dystrophy patients. *Mol. Ther. 21*, 1718−1726.

(30) Osborn, M. J., Starker, C. G., McElroy, A. N., Webber, B. R., Riddle, M. J., Xia, L., Defeo, A. P., Gabriel, R., Schmidt, M., Von Kalle, C., Carlson, D. F., Maeder, M. L., Joung, J. K., Wagner, J. E., Voytas, D. F., Blazar, B. R., and Tolar, J. (2013) TALEN-based gene correction for epidermolysis bullosa. *Mol. Ther. 21*, 1151−1159.

(31) Garg, A., Lohmueller, J. J., Silver, P. A., and Armel, T. Z. (2012) Engineering synthetic TAL effectors with orthogonal target sites. *Nucleic Acids Res. 40*, 7584−7595.

(32) Politz, M. C., Copeland, M. F., and Pfleger, B. F. (2013) Artificial repressors for controlling gene expression in bacteria. *Chem. Commun. 49*, 4325−4327.

(33) Lienert, F., Torella, J. P., Chen, J. H., Norsworthy, M., Richardson, R. R., and Silver, P. A. (2013) Two- and three-input TALE-based AND logic computation in embryonic stem cells. *Nucleic Acids Res.*, DOI: 10.1093/nar/gkt758.

(34) Perez-Pinera, P., Ousterout, D. G., Brunger, J. M., Farin, A. M., Glass, K. A., Guilak, F., Crawford, G. E., Hartemink, A. J., and Gersbach, C. A. (2013) Synergistic and tunable human gene activation by combinations of synthetic transcription factors. *Nat. Methods 10*, 239−242.

(35) Maeder, M. L., Linder, S. J., Reyon, D., Angstman, J. F., Fu, Y., Sander, J. D., and Joung, J. K. (2013) Robust, synergistic regulation of human gene expression using TALE activators. *Nat. Methods 10*, 243−245.

(36) Gossen, M., and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A. 89*, 5547−5551.

(37) Beerli, R. R., Dreier, B., and Barbas, C. F., III (2000) Positive and negative regulation of endogenous genes by designed transcription factors. *Proc. Natl. Acad. Sci. U.S.A. 97*, 1495−1500.

(38) Konermann, S., Brigham, M. D., Trevino, A. E., Hsu, P. D., Heidenreich, M., Cong, L., Platt, R. J., Scott, D. A., Church, G. M., and Zhang, F. (2013) Optical control of mammalian endogenous transcription and epigenetic states. *Nature 500*, 472−476.

(39) Li, Y., Moore, R., Guinn, M., and Bleris, L. (2012) Transcription activator-like effector hybrids for conditional control and rewiring of chromosomal transgene expression. *Sci. Rep. 2*, 897.

(40) Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) Toward controlling gene expression at will: Specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc. Natl. Acad. Sci. U.S.A. 95*, 14628−14633.

(41) Beerli, R. R., Schopfer, U., Dreier, B., and Barbas, C. F., 3rd. (2000) Chemically regulated zinc finger transcription factors. *J. Biol. Chem. 275*, 32617−32627.

(42) Magnenat, L., Schwimmer, L. J., and Barbas, C. F., 3rd. (2008) Drug-inducible and simultaneous regulation of endogenous genes by single-chain nuclear receptor-based zinc-finger transcription factor gene switches. *Gene Ther. 15*, 1223−1232.

(43) Schwimmer, L. J., Gonzalez, B., and Barbas, C. F., 3rd. (2012) Benzoate X receptor zinc-finger gene switches for drug-inducible regulation of transcription. *Gene Ther. 19*, 458−462.

(44) Pollock, R., Giel, M., Linher, K., and Clackson, T. (2002) Regulation of endogenous gene expression with a small-molecule dimerizer. *Nat. Biotechnol. 20*, 729−733.

(45) Dent, C. L., Lau, G., Drake, E. A., Yoon, A., Case, C. C., and Gregory, P. D. (2007) Regulation of endogenous gene expression using small molecule-controlled engineered zinc-finger protein transcription factors. *Gene Ther. 14*, 1362−1369.

(46) Beerli, R. R., and Barbas, C. F., III (2002) Engineering polydactyl zinc-finger transcription factors. *Nat. Biotechnol. 20*, 135−141.

(47) Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G., and Evan, G. I. (1995) A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res. 23*, 1686−1690.

(48) Vegeto, E., Allan, G. F., Schrader, W. T., Tsai, M. J., McDonnell, D. P., and O'Malley, B. W. (1992) The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell 69*, 703−713.

(49) Saez, E., Nelson, M. C., Eshelman, B., Banayo, E., Koder, A., Cho, G. J., and Evans, R. M. (2000) Identification of ligands and coligands for the ecdysone-regulated gene switch. *Proc. Natl. Acad. Sci. U.S.A. 97*, 14512−14517.

(50) Lamb, B. M., Mercer, A. C., and Barbas, C. F., III (2013) Directed evolution of the TALE N-terminal domain for recognition of all 5′ bases. *Nucleic Acids Res.*, DOI: 10.1093/nar/gkt754.

(51) Gaj, T., Guo, J., Kato, Y., Sirk, S. J., and Barbas, C. F., III (2012) Targeted gene knockout by direct delivery of zinc-finger nuclease proteins. *Nat. Methods 9*, 805−807.

(52) Tachikawa, K., Schroder, O., Frey, G., Briggs, S. P., and Sera, T. (2004) Regulation of the endogenous VEGF-A gene by exogenous designed regulatory proteins. *Proc. Natl. Acad. Sci. U.S.A. 101*, 15225−15230.

(53) Cantz, T., Key, G., Bleidissel, M., Gentile, L., Han, D. W., Brenne, A., and Scholer, H. R. (2008) Absence of OCT4 expression in somatic tumor cell lines. *Stem Cells (Dayton, Ohio) 26*, 692−697.

(54) Polstein, L. R., and Gersbach, C. A. (2012) Light-inducible spatiotemporal control of gene activation by customizable zinc finger transcription factors. *J. Am. Chem. Soc. 134*, 16480−16483.

(55) Khalil, A. S., Lu, T. K., Bashor, C. J., Ramirez, C. L., Pyenson, N. C., Joung, J. K., and Collins, J. J. (2012) A synthetic biology framework for programming eukaryotic transcription functions. *Cell 150*, 647−658.

(56) Mali, P., Aach, J., Stranges, P. B., Esvelt, K. M., Moosburner, M., Kosuri, S., Yang, L., and Church, G. M. (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol. 31*, 833−838.

(57) Perez-Pinera, P., Kocak, D. D., Vockley, C. M., Adler, A. F., Kabadi, A. M., Polstein, L. R., Thakore, P. I., Glass, K. A., Ousterout, D. G., Leong, K. W., Guilak, F., Crawford, G. E., Reddy, T. E., and Gersbach, C. A. (2013) RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat. Methods 10*, 973−976.

(58) Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., and Lim, W. A. (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell 152*, 1173−1183.

(59) Gilbert, L. A., Larson, M. H., Morsut, L., Liu, Z., Brar, G. A., Torres, S. E., Stern-Ginossar, N., Brandman, O., Whitehead, E. H., Doudna, J. A., Lim, W. A., Weissman, J. S., and Qi, L. S. (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell 154*, 442−451.

(60) Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F., and Marraffini, L. A. (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res. 41*, 7429−7437.

(61) Maeder, M. L., Linder, S. J., Cascio, V. M., Fu, Y., Ho, Q. H., and Joung, J. K. (2013) CRISPR RNA-guided activation of endogenous human genes. *Nat. Methods 10*, 977−979.

(62) Cheng, A. W., Wang, H., Yang, H., Shi, L., Katz, Y., Theunissen, T. W., Rangarajan, S., Shivalila, C. S., Dadon, D. B., and Jaenisch, R. (2013) Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res. 23*, 1163−1171.

(63) Farzadfard, F., Perli, S. D., and Lu, T. K. (2013) Tunable and multifunctional eukaryotic transcription factors based on CRISPR/ Cas. *ACS Synth. Biol.*, DOI: 10.1021/sb400081r.

(64) Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J. A., Somia, N. V., Bogdanove, A. J., and Voytas, D. F. (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res. 39*, e82.