

Small-molecule-based regulation of RNA-delivered circuits in mammalian cells

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Synthetic mRNA is an attractive vehicle for gene therapies because of its transient nature and improved safety profile over DNA. However, unlike DNA, broadly applicable methods to control expression from mRNA are lacking. Here we describe a platform for small-molecule-based regulation of expression from modified RNA (modRNA) and self-replicating RNA (replicon) delivered to mammalian cells. Specifically, we engineer small-molecule-responsive RNA binding proteins to control expression of proteins from RNA-encoded genetic circuits. Coupled with specific modRNA dosages or engineered elements from a replicon, including a subgenomic promoter library, we demonstrate the capability to externally regulate the timing and level of protein expression. These control mechanisms facilitate the construction of ON, OFF, and two-output switches, with potential therapeutic applications such as inducible cancer immunotherapies. These circuits, along with other synthetic networks that can be developed using these tools, will expand the utility of synthetic mRNA as a therapeutic modality.

Gene therapy has the potential to revolutionize healthcare, using genetic material to deliver therapeutic components and treat disease. Recently, adeno-associated virus (AAV) and retrovirus-based gene therapies have demonstrated curative effects in the clinic via long-lasting gene expression^{1–3}. However, for applications that benefit from transient protein expression, such as gene editing, vaccination, and cellular reprogramming, transfection with short-lived synthetic nucleic acids may be more appropriate⁴. A particularly attractive option is synthetic mRNA that is transcribed in vitro and then delivered to cells. Synthetic mRNA has an improved safety profile compared to synthetic DNA; it possesses virtually no risk of genomic integration and degrades via intracellular mechanisms⁴. Synthetic mRNAs incorporating base modifications (modRNA) have been shown to have decreased immunogenicity and increased expression compared to traditional unmodified mRNAs^{5,6}. Depending on the desired application, transgenes can be expressed from modRNA⁴ or from self-replicating RNAs (replicons), which are more immunogenic but provide high levels of prolonged expression^{7,8} (see Supplementary Fig. 1 for a description of replicons).

Lack of control is one of the primary concerns for existing gene therapies, as current therapeutics rely almost exclusively on constitutive gene expression⁹. One example of clinical relevance is AAV-based treatment of hemophilia. The expression of factor VIII or IX proteins from DNA delivered to the liver can prevent or reduce bleeding in hemophilia A or B patients, respectively^{2,3}. However, supraphysiological expression levels of factor VIII or IX in the body may put the patient at an increased risk of clotting and thromboembolisms^{10,11}. Furthermore, great care must be taken when a protein

with a narrow therapeutic index is expressed from a gene therapy vector. For instance, recombinant interleukin-12 has demonstrated some early clinical efficacy in treating cancer but was highly toxic when administered intravenously at high concentrations (in the absence of an initial priming dose), leading to several instances of treatment-related patient deaths¹².

For DNA, well-established methods to control the strength and timing of transgene expression using exogenous small molecules exist¹³ and may be used to improve the safety and efficacy of gene therapies, such as those described above. However, for in vitro-transcribed RNA, similar control over expression has remained elusive. Aptazymes, encoded and delivered in the form of DNA, have been shown to regulate RNA transcribed inside a cell (for example, in yeast¹⁴). Yet, to our knowledge, aptazymes are currently incompatible with in vitro-transcribed RNAs because they are activated under standard in vitro transcription conditions, causing cleavage and inactivation of the RNA before delivery into cells. Researchers have also devised various small-molecule-responsive degradation domains to control protein stability^{15–17}. Although one type of degradation domain has been fused to the measles virus (MeV) P protein to regulate the replication of MeV¹⁷, an RNA virus, regulation of a replicase protein is not generally applicable to all types of RNA (for example, modRNA). Moreover, a general caveat of these degradation-domain-based approaches is that the protein of interest must be fused directly with these degradation domains. These fusions can potentially compromise the function of the protein. Furthermore, these fusions may not be able to modulate protein stability well in situations wherein the protein is not accessible to the relevant proteases that interact with these domains (for example, for secreted

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proteins)¹⁸. Previously, we developed synthetic RNA circuits whose activity is regulated with exogenously administered small interfering RNA (siRNA) inputs¹⁹. However, for the purpose of regulating synthetic mRNA circuits in a clinical setting, siRNA may be impractical because it is currently not possible to target organs other than the liver efficiently in vivo²⁰, and siRNA has been associated with unacceptable toxicity in some clinical trials²¹. Additionally, siRNA regulation relies on degradation of part of the RNA circuit and thereby prevents reversibility and dynamic control in RNA-only platforms.

To address these challenges, we developed novel small-molecule-responsive RNA binding proteins (RBPs) to generate externally controlled RNA circuits. Though dose alone could tune the performance of modRNA circuits, novel viral RNA elements, including a subgenomic promoter library and additional 3' UTR sequences, were required for replicons. Together, these mRNA regulatory devices were used to precisely control expression of multiple proteins from in vitro-transcribed synthetic RNA directly transfected into mammalian cells. We then demonstrated their potential in both a modRNA and replicon platform by building small-molecule-responsive ON, OFF, and two-output switches.

Results

Regulating modified RNA circuits using small molecules. To build a general platform for regulated expression from synthetic RNA, we first implemented small-molecule-responsive gene circuits delivered with modRNA²². Specifically, we controlled gene expression from modRNA by fusing RBPs to destabilization domains (DDs), which are targeted for degradation by the proteasome unless the DD is stabilized by a cognate small molecule¹⁵. To confirm DD function, we fused the fluorescent protein mVenus to a DD engineered from the *Escherichia coli* dihydrofolate reductase²³ (referred to hereafter as DDd) and expressed it from modRNA. DDd is stabilized by trimethoprim (TMP), an FDA-approved small-molecule antibiotic, and the addition of TMP stabilized DDd-mVenus as expected (Supplementary Fig. 2). Because these DD fusions may disrupt a protein's native function or affect intracellular localization²⁴, we next demonstrated that we can control a protein's abundance indirectly by using an RBP. To this end, we created an OFF switch by fusing DDd to L7Ae, a ribosomal protein from *Archaeoglobus fulgidus* that binds RNA kink-turn (k-turn) motifs with high affinity²⁵. Placing k-turns in the 5' UTR of an mRNA allows L7Ae to repress expression of the downstream open reading frame (ORF)¹⁹ (Supplementary Fig. 3). To test the system, we co-transfected two modRNAs, with the first encoding a DDd-L7Ae fusion protein and the second an mVenus reporter with two k-turns in its 5' UTR. In the absence of TMP, DDd destabilized the L7Ae repressor and allowed expression of the mVenus reporter (ON state; Fig. 1a and Supplementary Fig. 4). Upon addition of TMP, DDd-L7Ae was stabilized and repressed expression of the reporter (OFF state). DDd-L7Ae was coupled to a fluorescent mKate reporter using a ribosome-skipping 2A sequence to indirectly monitor translation of DDd-L7Ae. By titrating the relative amounts of repressor and reporter modRNA, we were able to tune performance and increase ON/OFF ratios, obtaining a range from 1.4-fold to 9.3-fold (Fig. 1a).

As opposed to an OFF switch, an ON switch functions via induction of expression. With this topology, an external small-molecule input is required only when the regulated protein is needed in the system. Such circuits are useful when expressing proteins with possible toxic effects, because a constant supply of small molecule is not required to prevent protein expression as it would be with an OFF switch. To engineer this circuit topology, we adapted the tetracycline-responsive repressor TetR. Though TetR is commonly known as a transcription factor that binds DNA²⁶, it also binds tightly to RNA aptamers in a manner that is sensitive to tetracycline and its derivatives such as doxycycline (Dox)²⁷. We encoded TetR aptamers

in the 5' UTR of a fluorescent reporter, expecting that in the absence of Dox, TetR would bind the aptamer and repress translation, which would be relieved when Dox was introduced. However, in preliminary experiments using modRNA, we observed a negligible response to Dox administration (Supplementary Fig. 5). To make the system more responsive, we fused TetR to the mammalian dead box helicase 6 (DDX6) protein. DDX6 interacts with the CCR4-NOT RNA degradation-silencing complex, and its homologs were shown to enhance TetR/aptamer-mediated translational repression in *Saccharomyces cerevisiae* and *Plasmodium falciparum*²⁸. By adjusting the initial doses of repressor and reporter, we improved the response of the ON switch from 1-fold to 4-fold (Fig. 1b and Supplementary Fig. 6). We next created a two-output switch, which supports the use of inducers for choosing between one of two gene expression states. We created a two-output switch by connecting TetR-DDX6 to the DDd-L7Ae switch, such that TetR-DDX6 repressed DDd-L7Ae, which in turn repressed the reporter mVenus. We achieved an ON/OFF response of 10.8-fold and 2.8-fold for mVenus and mKate, respectively (Fig. 1c and Supplementary Fig. 7).

Engineering control elements for replicons. We next sought to regulate gene expression from replicons using approaches similar to those described above for modRNA. For this purpose, we use a replicon derived from Venezuelan Equine Encephalitis (VEE) virus, a well-characterized positive-strand RNA virus of the alphavirus genus, which has been used for several applications including reprogramming²⁹ and vaccination³⁰. Our experience with modRNA highlighted the importance of adjusting the relative expression of circuit components to optimize circuit performance. However, although the level of gene expression from modRNA can be modulated by varying the transfected dose of RNA (Supplementary Fig. 8), we have shown previously that expression from an alphavirus replicon reaches a saturation point that is independent of the initial dose³¹. Furthermore, although we also demonstrated that co-transfection of several replicons can be used to predictably control the expression levels of multiple proteins over short time periods³¹, such regulation was not stable over longer periods because one replicon species outcompeted the other(s)¹⁹. Hence, we needed an approach that allowed independent regulation of multiple proteins from a single replicon molecule.

We began by engineering elements within the VEE replicon genome to predictably regulate the expression of multiple genes from a single replicon. We first focused our efforts on engineering replicon subgenomic promoters (SGPs), which initiate transcription of the subgenomic RNA used to express heterologous proteins. Multiple SGPs have been used previously to express more than one protein from a replicon³², and studies of the SGP of an alphavirus demonstrated that truncating SGPs could affect subgenomic expression levels³³. However, systematic efforts to precisely tune expression from SGPs have not been made. Thus, we generated a library of 27 SGPs by truncating the +30 bp region of the wild-type SGP (the subgenomic transcription initiation site is defined as the +1 position of the SGP). We engineered SGPs with up to a 15.8-fold difference in expression using this approach (Fig. 2a and Supplementary Fig. 9). Hereafter, truncated SGPs use the -98 bp of the wild-type SGP (see Methods) and are named according to the number of bases remaining on the (+)-side of the wild-type SGP; for example, a truncated promoter that retains -98/+5 bp of the wild-type SGP is referred to as SGP5. To enable an efficient methodology for construction of complex replicon circuits, we developed a hierarchical cloning strategy based on the MoClo method that was used to build all replicons described below (Supplementary Fig. 10)³⁴.

From the SGP truncation library characterization, we observed that SGP30 at the first position expressed the reporter at levels 11.9 times lower than the same SGP30 sequence in the second position. Though such positional effects could be useful when designing

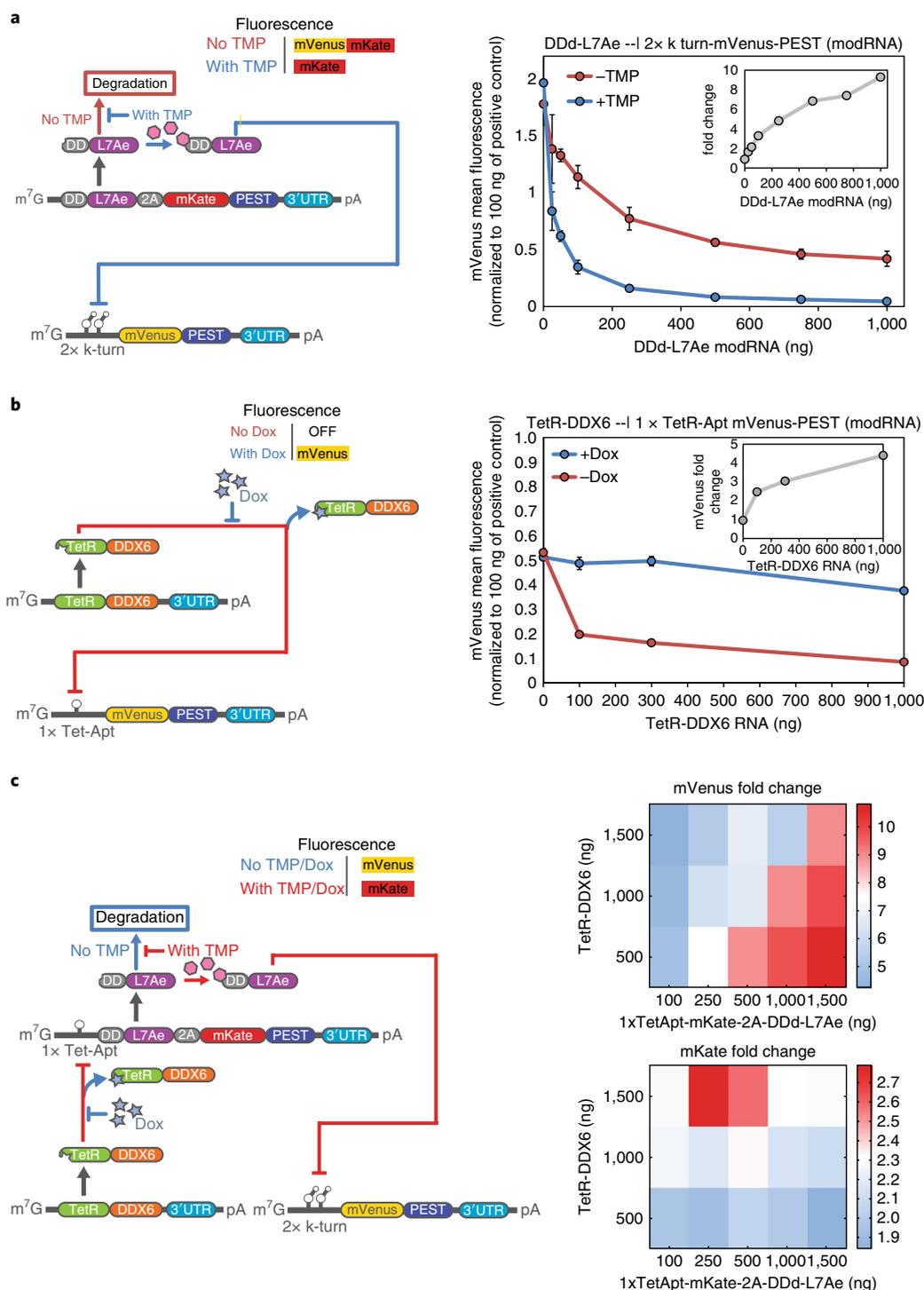


Fig. 1 | Small-molecule-based regulation of expression from modRNA. a, To generate an OFF switch, two modRNAs were co-transfected, with one expressing mKate-PEST-2A-DDd-L7Ae and the other 2x k-turn-mVenus-PEST. As DDd-L7Ae RNA doses increased, mVenus expression levels for both TMP states decreased. In the absence of TMP, incomplete degradation of L7Ae by DDd results in a response to DDd-L7Ae dose. However, a more marked increase in repression is found when TMP is present, resulting in increasing fold changes (–TMP/+TMP) for higher DDd-L7Ae doses. **b**, An ON switch was created by co-transfecting two modRNAs expressing TetR-DDX6 and 1x TetR-aptamer-mVenus-PEST. In the absence of Dox, TetR-DDX6 binds the TetR aptamer (Tet-Apt) and represses mKate. When Dox is introduced, it binds TetR and prevents binding of TetR to the aptamer, resulting in mKate expression. Fold changes shown are +Dox/–Dox. **c**, A two-output switch was created by coupling the ON and OFF switches. Three modRNAs, expressing TetR-DDX6, 1x TetR-aptamer-mKate-PEST-2A-DDd-L7Ae, and 2x k-turn-mVenus-PEST, were co-transfected. The dose of 2x k-turn-mVenus-PEST was held constant at 1,000 ng. In the absence of Dox and TMP, TetR-DDX6 binds the TetR-aptamer and represses mKate and DDd-L7Ae. DDd-L7Ae expression levels are further decreased by destabilization and degradation, resulting in high mVenus levels. When Dox and TMP are both introduced, TetR-DDX6 is inhibited and DDd-L7Ae is stabilized, resulting in high mKate and low mVenus expression levels. By coupling these two small-molecule-responsive RBPs, we achieved a 10.8-fold change in mVenus. All results were normalized to cells transfected with 100 ng of either SGP30-mVenus or SGP30-mKate modRNA, used as standards. Error bars represent s.d. of the geometric mean with $n \geq 2$.

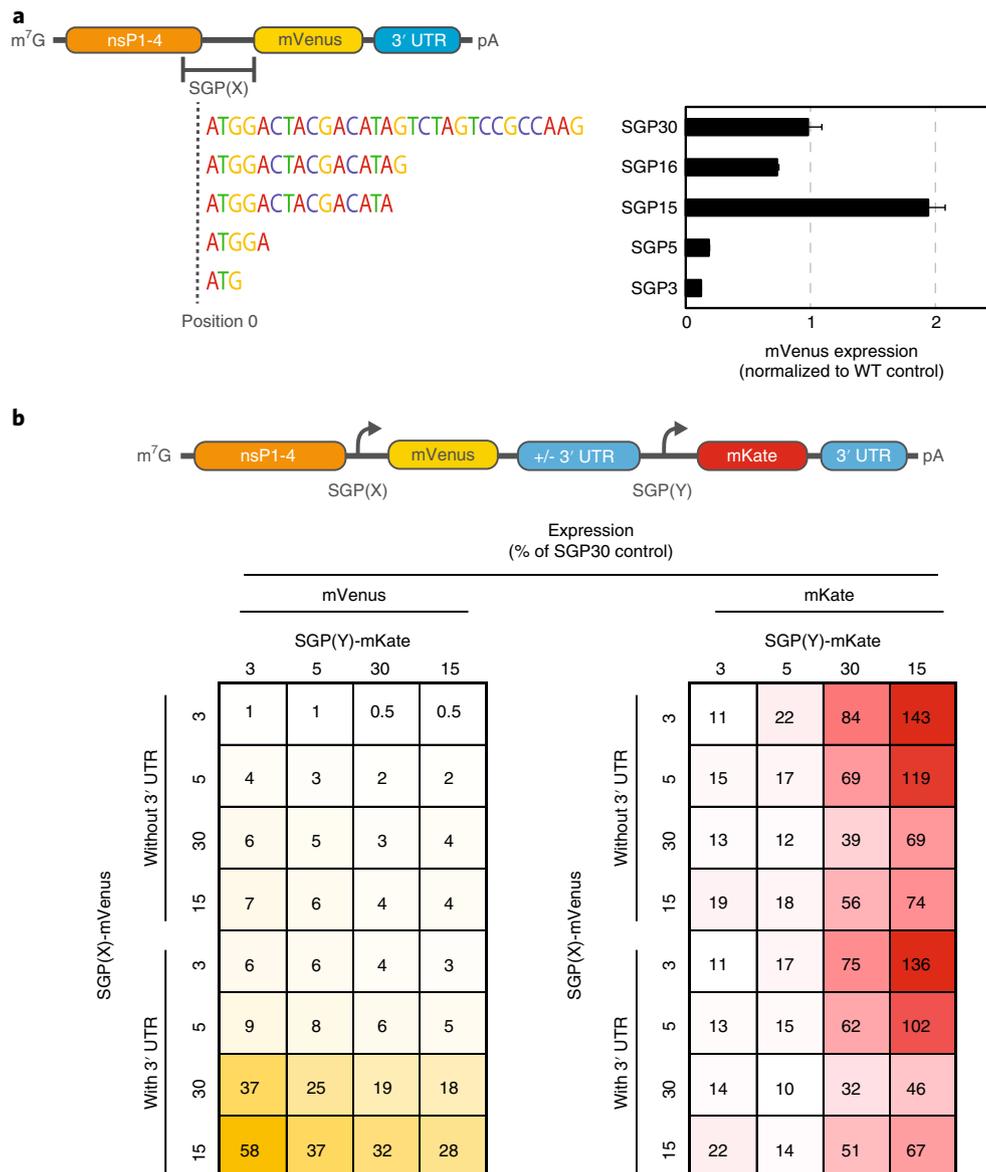


Fig. 2 | Small-molecule-based regulation of expression from replicons. a, An SGP truncation library enables tuning of expression from subgenomic RNA. Deletions to the +30 region of the wild-type SGP result in a 16-fold range in expression levels for a single SGP replicon. **b**, Characterization of two SGP replicons demonstrates the impact of position, SGP strength, and 3' UTR sequence on expression. All data were normalized to cells transfected with 1 μ g of single SGP30-reporter replicons expressing either mVenus or mKate. Fluorescence measurements were taken 24 h post-transfection, and error bars represent s.d. of the geometric mean with $n \geq 2$.

certain gene circuits, increasing expression from the upstream SGP would enhance our ability to both increase overall replicon expression levels and better match expression with downstream SGPs. In other positive-strand RNA viruses, proximity to a 3' UTR has been shown to increase protein expression³⁵. Thus, to increase the proximity of the first ORF to a 3' UTR, we inserted an additional 3' UTR sequence downstream of SGP30-mVenus in the first position and observed a six-fold increase in mVenus expression (Supplementary Fig. 11a,b). To further understand positional effects and the impact of additional 3' UTR sequences, we created a library of 32 two-SGP replicons with combinations of very low (SGP3), low (SGP5), medium (SGP30), and high (SGP15) strength SGPs with and without an additional 3' UTR sequence following mVenus. We found that expression of the downstream gene was always stronger than expression of the upstream gene when using identical SGPs, and we observed a 300-fold difference between expression

under the weakest SGP in the first (upstream) position compared to the strongest SGP in the second (downstream) position (Fig. 2b and Supplementary Fig. 11). We also confirmed that including an additional 3' UTR sequence led to increased mVenus expression. Stronger SGPs saw more pronounced increases, and qRT-PCR confirmed that this increase in reporter expression was due to an increase in the corresponding subgenomic RNA (Supplementary Fig. 11c,d). Having identified that SGP sequence length, position, and 3' UTR sequences are critical parameters affecting expression, we explored their combined effect on gene expression in a library of three SGP replicons comprising fluorescent protein reporters at each position (Supplementary Fig. 12). The results demonstrate a consistent relationship between SGP strength, position, 3' UTR sequence, and relative expression level of the genes in a multi-expression unit replicon, which is useful for circuit design and other expression tuning objectives.

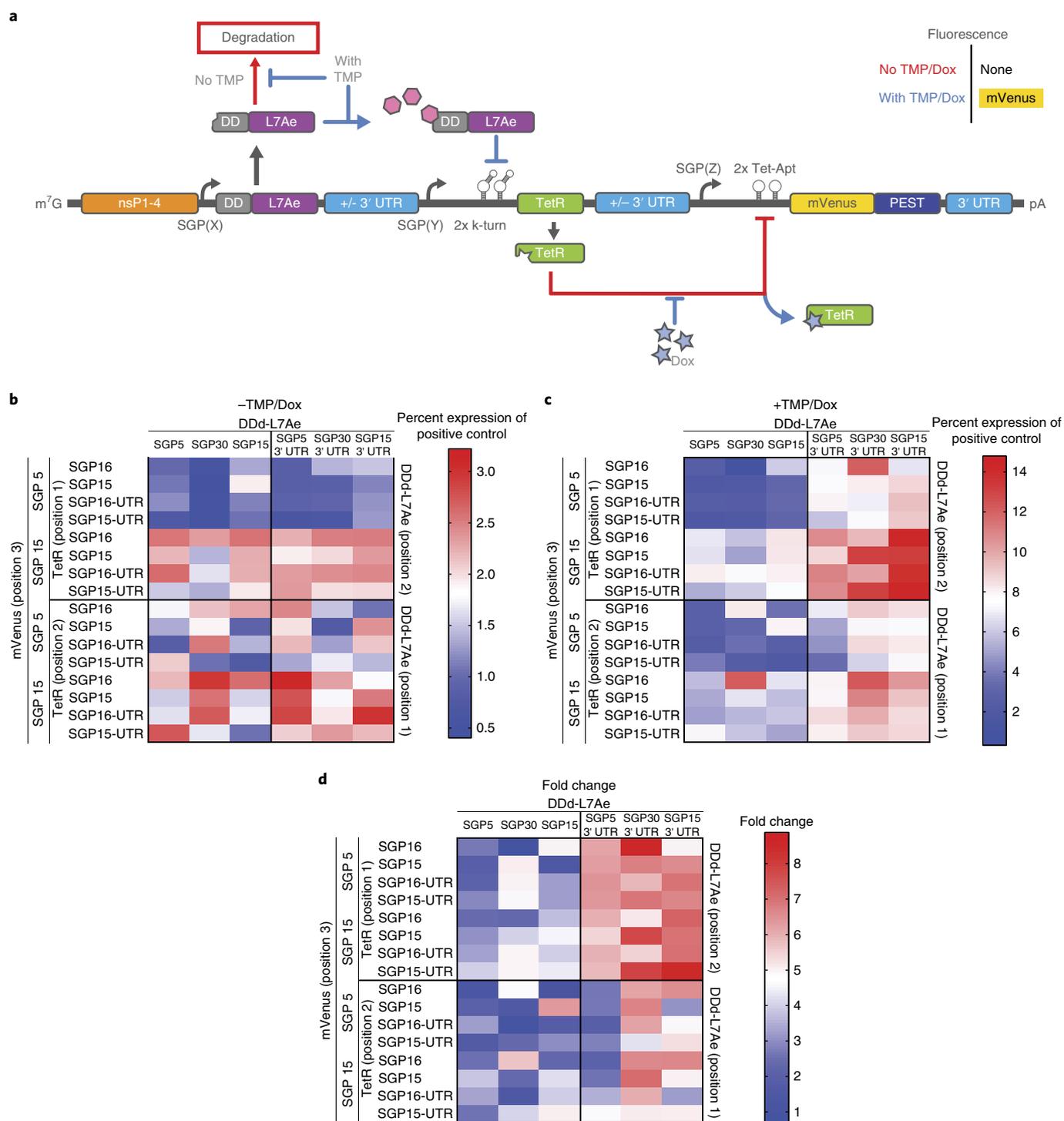


Fig. 3 | Small-molecule-based ON switches driven from replicons. **a**, A TMP/Dox-responsive ON switch was created using a cascade of DDd-L7Ae and TetR repressors. Ninety-six variants of this circuit were created by modulating the position, SGP strength, and 3' UTR sequence of DDd-L7Ae and TetR. Here, we show DDd-L7Ae in the first position and TetR in the second. When TMP is absent, DDd-L7Ae is degraded, allowing TetR to repress mVenus expression. When TMP is introduced, DDd-L7Ae is stabilized, repressing TetR and allowing mVenus expression. Dox is also added in the + TMP state to further inhibit TetR. **b–d**, The heat maps shown reflect the OFF state (**b**), ON state (**c**), and fold change (**d**) 48 h post-transfection. Results were normalized to cells transfected with 1 μ g replicon encoding SGP30-mVenus, and geometric mean was taken with $n \geq 2$.

Designing small-molecule-responsive replicon switches. Using our new SGP and 3' UTR toolbox, we next sought to build small-molecule-responsive switch circuits that could control the expression of unmodified proteins from single replicons. We first showed that L7Ae and TetR are both capable of repressing an mVenus

reporter located on the same replicon (Supplementary Fig. 13) and that DDd-mVenus expression increases 5.1-fold upon addition of TMP (Supplementary Fig. 14). We then built a library of 12 replicon-based OFF switches using DDd-L7Ae and a variety of SGP strengths (Supplementary Figs. 15 and 16). We discovered that

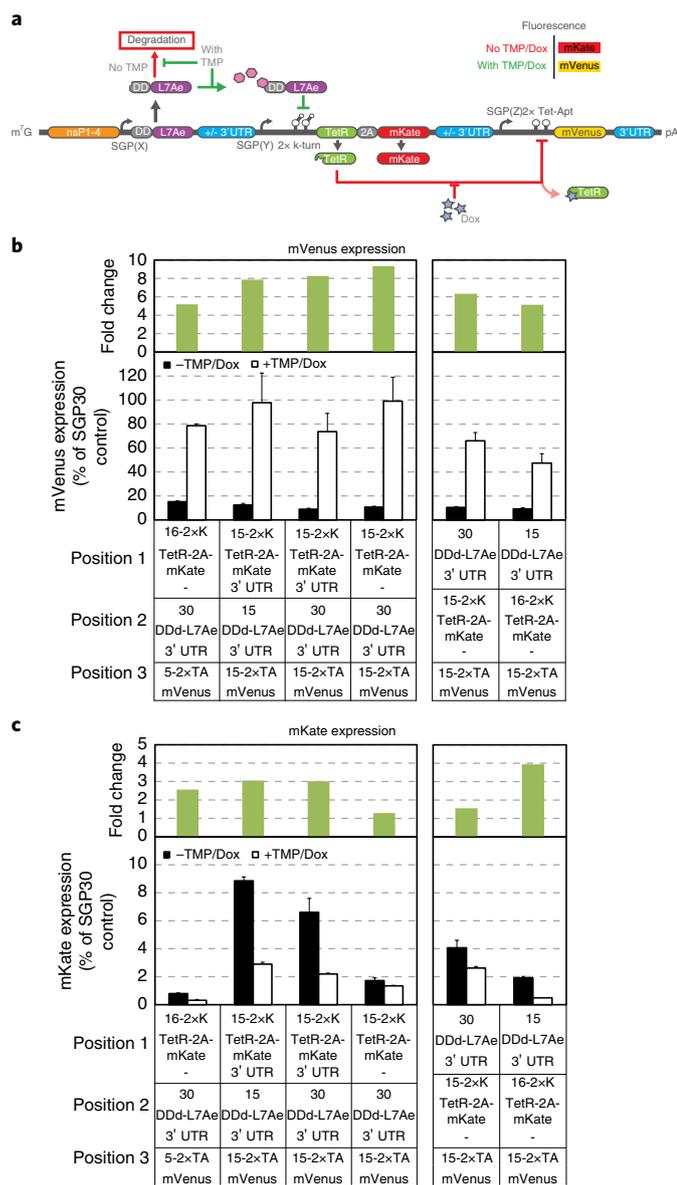


Fig. 4 | Small-molecule-based two-output switches driven from replicons. **a**, A two-output switch design incorporates mKate into the top performing ON switch circuit configurations from Fig. 3. When TMP is absent, DDd-L7Ae is degraded, allowing mKate expression and TetR to repress mVenus expression. When TMP is introduced, DDd-L7Ae is stabilized, repressing mKate and TetR, and allowing mVenus expression. Dox was also added in the + TMP state to further inhibit TetR. **b, c**, For these top performing circuits, we observed a 5.1- to 9.3-fold change in mVenus expression (**b**) and a 1.2- to 3.9-fold change in mKate expression (**c**) upon addition of TMP/Dox. In each of these circuits, DDd-L7Ae is driven by strong SGPs (SGP15 or SGP30), and all six include an additional 3' UTR to increase expression (here, 2xK and 2xTA are abbreviations of 2x k-turn and 2x TetR-aptamer, respectively). Note that inserting a 2A-mKate may alter TetR expression compared to the circuits in Fig. 3 because of incomplete 2A cleavage or increasing subgenomic RNA length. Additionally, PEST sequences were removed to increase the level of mKate expression when driven from the first subgenomic promoter due to positional effects. All data were normalized to single SGP30-reporter controls expressing either mVenus or mKate. Fluorescence measurements were taken 48 h post-transfection, and error bars represent s.d. of the geometric mean with $n \geq 2$.

increasing the strength of the SGP driving DDd-L7Ae from the first position influences the fold-change between repressed and derepressed states (maximum 16-fold change).

To further enhance the ability to regulate expression from replicons, we next sought to create a replicon-encoded small-molecule ON switch. An initial circuit architecture using TetR/Dox regulation resulted in little-to-no change in expression upon addition of Dox (Supplementary Fig. 17). We revised the circuit by again taking advantage of DDX6, as we did with modRNA. Fusing DDX6 to TetR resulted in a small-molecule-regulated ON switch with 19-fold increase in expression when 1 μ g/ml of Dox was introduced (Supplementary Fig. 18).

We then focused on creating a two-output switch using the same topology described for modRNA (Fig. 1c). Our initial circuit optimization was performed using a single output ON switch comprising DD-L7Ae regulating expression of TetR, which in turn repressed a reporter (Fig. 3; Supplementary Figs. 19 and 20). In the absence of TMP, DDd-L7Ae is degraded, allowing TetR to be translated and repress the expression of the fluorescent reporter mVenus. If TMP is present, however, DDd-L7Ae represses TetR, enabling expression of the reporter. The addition of Dox helps reporter expression. Based on our observation of the effect of position on constitutive expression, we placed the mVenus reporter under the third SGP to maximize expression. We then evaluated a library of 96 circuit variants in which we permuted the positions of DDd-L7Ae and TetR, the SGP variant controlling their expression, and the presence or absence of a 3' UTR; the best-performing circuit demonstrated an 8.9-fold change upon addition of both TMP and Dox (Fig. 3; Supplementary Figs. 19 and 20). The results were consistent with those from the modRNA switches and the replicon-encoded DDd-L7Ae OFF switch: altering DDd-L7Ae expression levels had a greater impact on circuit function than changing TetR levels, with higher expression of DDd-L7Ae typically resulting in increased circuit performance and fold change (Fig. 3; Supplementary Figs. 19 and 20). Both the ON state mVenus expression level and fold change are primarily determined by the DDd-L7Ae expression levels. Moreover, such expression levels would not have been attainable without introducing additional 3' UTR sequences, demonstrating their pivotal role in developing more complex RNA circuits. The five top-performing circuit configurations contained DDd-L7Ae under the second SGP with a 3' UTR, increasing its expression and leading to lower TetR expression from the first SGP. To create a two-output switch, we attached a ribosome-skipping 2A sequence and a fluorescent mKate reporter to TetR for the top six performing circuit configurations (Fig. 4 and Supplementary Fig. 21). We observed a 5.1- to 9.3-fold change in mVenus expression and a 1.2- to 3.9-fold change in mKate expression upon the addition of TMP and Dox.

Using small molecules as circuit inputs also enables concentration-dependent and dynamic control over gene expression from these synthetic RNA circuits. To this end, we show that the duration and temporal sequence of small-molecule induction correlates with expression levels for different circuit topologies (Supplementary Figs. 22 and 23). Such ability to externally control the magnitude and timing of gene expression from modRNA or replicons using small molecules could have important therapeutic implications, as discussed below. Additionally, although this work was initially developed in BHK-21 cells, to demonstrate its general applicability we also validated a subset of the aforementioned circuits in a mouse myoblast cell line (C2C12; Supplementary Fig. 24).

Discussion

The field of synthetic biology has established a regulatory framework for DNA-based therapies using inputs ranging from external small molecules to protein biomarkers of various diseases⁹. As DNA-driven gene and cell therapies become more commonplace,

next-generation versions of such therapies may incorporate regulatory motifs for more precise control of therapeutic activities. Similarly, mRNA-based therapeutics may also benefit from such control mechanisms. However, for mRNA, a general approach to regulate protein expression using small molecules had not been established until now. In this report, we describe a platform for controlling protein expression from synthetic mRNA by combining small-molecule-responsive RNA binding proteins with modRNA technology and replicon engineering. We demonstrated the use of Ddd-L7Ae and TetR as small-molecule-responsive RBPs to develop ON, OFF, and two-output switches. To optimize the performance of these circuits, we modulated the expression of each circuit component using modRNA titration and a library of SGPs and 3' UTR sequences. We have characterized each of the regulatory elements independently and in concert to demonstrate their effect on protein expression and circuit function, creating a toolbox of regulatory elements that can be used to precisely control protein expression from synthetic mRNA.

The ability to externally control the magnitude and timing of gene expression from the switches presented here could have important therapeutic implications. As more mRNA therapies enter the clinic, small-molecule-based control of protein expression may become necessary for patient safety in addition to drug efficacy. For instance, a modRNA ON switch may improve the safety of genome editing applications by preventing prolonged expression of a nuclease and decreasing its potential for off-target editing via transient administration of the small-molecule input. Additionally, a modRNA or replicon OFF switch could be critical for the expression of immune effector proteins, which should be continuously expressed except for during infrequent events when downregulation is required to prevent cytotoxicity. Finally, dynamic and reversible control of protein expression from replicons could be a key enabler of programmable vaccines, for which timing of antigen expression would be regulated via small-molecule drug administration³⁶.

However, several challenges remain in the translation of this technology to the clinic. One possible caveat of these approaches is the possibility that the non-native protein components used in the circuit may trigger an immune response in the patient's body. Replicons, in particular, encode viral nonstructural proteins that may trigger an adaptive immune response³⁷; however, we have observed strong reporter protein expression upon repeated injection of lipid nanoparticle-encapsulated replicon in mice, suggesting the possibility to overcome this limitation (unpublished results in collaboration with D. Irvine's group at MIT). Nevertheless, mechanisms to prevent such immune responses, such as prophylactic corticosteroid usage or tolerance induction, may be necessary to actually deploy these circuits in the clinic^{38–40}. Furthermore, replicons are known to induce a potent innate immune response because of stimulation of intracellular pattern recognition receptors and subsequent type I interferon induction, potentially limiting its applications to immunological therapies⁷. Although modRNA is far less immunogenic, its inability to self-amplify may lead to other limitations in vivo, including limited duration and strength of gene expression.

Our platform also has the potential to be enhanced by other relevant work on regulating mRNA or protein expression. For example, additional RBPs can be coupled to other DDs¹⁵ with orthogonal small molecules to generate more complex multi-input circuits. The discovery of new endoRNases⁴¹ opens a door to a completely novel set of circuits, for example, a small-molecule-regulated safety switch that cleaves the RNA circuit when a small molecule is introduced. modRNA and replicons are also attractive platforms for ancillary regulatory mechanisms, such as microRNA (miRNA) targeting, which would restrict circuit function to cells with the correct miRNA profile¹⁹. We hope that the circuits described in this report

may serve as an inspiration for future small-molecule-regulated mRNA therapeutics.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41589-018-0146-9>.

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Author contributions

J.R.B., T.W., K.B., and T.K. designed and performed experiments and analyzed data. X.Z., A.W., E.P., and B.D. performed experiments and interpreted data. O.A., N.N.S., and D.D. designed experiments. B.A. performed experiments. J.B. analyzed data. T.K. and R.W. supervised the study. T.W., J.R.B., B.T., T.K., and R.W. wrote the manuscript with the support of all other authors.

Competing interests

MIT has filed a patent application (No. 15/509,258) pertaining to the technology described in this paper. T.W., J.R.B., K.B., T.K., and R.W. are co-inventors on this patent application. J.R.B. and T.K. are shareholders of a company founded based on the technology described in this article.

Additional information

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Methods

Replicon cloning strategy. A VEE replicon was derived previously¹⁹ from the TC-83 strain, containing an A3G mutation as well as a Q739L mutation in nsP2. This replicon vector was adapted for use with modular cloning (MoClo) to enable flexible and hierarchical assembly of constructs^{34,42}. MoClo assembly allowed us to efficiently clone and characterize large numbers of replicons with a diverse set of control elements, including RBPs, SGPs, and 3' UTRs. Each subgenomic translational unit of a replicon was divided into three parts: an SGP, ORF, and 3' UTR (Supplementary Fig. 25). To reduce the length of the final replicon, a minimal SGP was used that retained full activity. As shown in Supplementary Fig. 9, truncating the minus side of the SGP begins to affect expression between $-61/+30$ and $-51/+30$. To include an additional buffer sequence, we set the minus side length of standard SGP parts at -98 base pairs, so only plus-side truncation would affect expression levels (-98 base pairs was also the minimal sequence requirement for full activity in Sindbis replicons⁴³). Note that the replicon backbone vector is designed so that once the construct is fully assembled, the 5' most SGP recreates the original wild-type sequence of the virus (for example, it is not truncated at -98). SGP, ORF, or 3' UTR parts were each separately cloned into Level 0 vectors, which contain BsaI recognition sites flanking the insert. BsaI, a Type IIS restriction enzyme, recognizes a sequence and cleaves downstream of its recognition site, allowing for scarless assembly. Level 0s were assembled into a Level 1 destination vector to form a single translational unit, using conserved sequences in between the SGP, ORF, and 3' UTR. Finally, Level 1s were inserted in a position-dependent manner into the replicon backbone using a second Type IIS enzyme, SapI, forming the final Level 2 product: a functional (multi-unit) replicon. Golden gate assembly reactions were performed using a standard protocol by mixing 50 ng of backbone vector with equimolar amounts of each insert, 1 μ l of 10 \times T4 DNA ligase buffer (Promega), 0.1 μ l of 100 \times BSA (NEB), 0.5 μ l of either BsaI-HF or SapI (NEB), 0.5 μ l of T4 DNA ligase (high concentration; Promega), and water up to 10 μ l. Reaction conditions were 1 h at 37 °C, 5 min at 50 °C, and 5 min at 80 °C. For constructs with internal BsaI or SapI sites, T4 ligase was added following inactivation at 80 °C and a final ligation step at 16 °C for 10 min was included.

Sequence level descriptions of the Replicon MoClo Assembly vectors, beginning with Level 0 destination vectors, are presented in Supplementary Fig. 26. These Level 0 destination vectors were originally made for use with either of the following Type IIS enzymes: SapI or BbsI. However, in practice, BsaI sites were used instead to reduce the time to cloning of the final construct (explained below). SapI has a 7-base-pair (bp) recognition site and a 3-bp overhang, whereas BbsI has a 6-bp recognition site and a 4-bp overhang. If new parts contained BsaI or SapI sites, these sites were mutated via a degenerate codon swap to make the Level 0 \rightarrow 1 and Level 1 \rightarrow 2 reactions more efficient, respectively, but this is not required if a final ligation step is added to the MoClo reaction. Our SGP library and the VEE 3' UTR do not contain recognition sites for either of these enzymes, so this task most commonly arises with ORFs. However, restriction sites should be considered when incorporating any new part, such as an aptamer sequence or modified 3' UTR. The Level 0 destination vectors contain ampicillin resistance (AmpR) cassettes, with the BsaI site in the AmpR gene mutated to facilitate a more efficient reaction of Level 0 \rightarrow 1. In addition, we mutated the BsaI site in the *cadB* gene to facilitate Level 0 generation via a digest/ligation reaction using BsaI with the same reaction conditions; this reaction was very efficient because the *cadB* gene killed the transformed bacteria that did not receive the insert. Generally, this was how we introduced new ORFs as stated above, because, if flanked by BsaI sites via PCR, the ORF could be incorporated into Level 0 (for future use) and Level 1 (to remove a cloning step) vectors at the same time.

After a library of SGPs, ORFs, and 3' UTRs was established, Level 0s were combined to make Level 1s (kanamycin resistance), which are individual translational units (Supplementary Fig. 27). As we have shown, position on the replicon has a significant effect on expression, and therefore the Level 1 destination vectors were designed to designate the translational unit's position in the final construct. In addition, some units have 3' UTR sequences, whereas others do not. Finally, the most 3'-translational unit always concluded with a truncated E1 protein followed by a 3' UTR sequence. This truncated E1 protein was present in the VEE replicon previously used¹⁹ and was included in all replicons used herein. These constraints left us with seven Level 1 destination vectors (Supplementary Fig. 27).

Notice that to assemble a single-SGP replicon, this strategy is cumbersome, requiring two rounds of reactions: first combining SGP, ORF, and E1-3' UTR into a Level 1 and then inserting this single translational unit into a Level 2. However, when characterizing a new ORF, for example, it may be beneficial to test and observe expression driven from a single SGP before constructing more complex multi-SGP replicons. To speed up cloning for single gene replicons, we created Level 0-S vectors (Supplementary Fig. 28). These vectors are similar to Level 1 vectors (kanamycin resistance) but contain either an SGP, ORF, or 3' UTR rather than an entire translational unit. Level 0-S vectors can be combined directly into a Level 2 vector to test specific expression levels of an ORF before more in-depth characterization. After such characterization, the Level 0-S can be transferred to Level 0 (using SapI) for use with the Replicon MoClo Assembly. Finally, Level 1 or Level 0-S plasmids can be combined into a Level 2 replicon backbone with ampicillin resistance (Supplementary Fig. 27).

RNA generation. VEE replicon *in vitro* transcription (IVT). VEE replicon plasmids constructed using the MoClo assembly strategy were linearized using I-SceI (NEB) and purified with QIAquick PCR Purification Kit (Qiagen) before run-off IVT. IVTs were performed using either the mMACHINE mMachine T7 Kit (Life Technologies) or the MEGAscript T7 Transcription Kit (Life Technologies) at 37 °C for 4 h. The resulting RNA was DNase treated and purified using either the RNeasy Mini Kit (Qiagen) or lithium chloride precipitation. For lithium chloride precipitation, 1/2 volume of ice-cold LiCl solution (7.5 M LiCl, 50 mM EDTA) was added to the IVT RNA and incubated at -20 °C for 30 min. After incubation, the mixture was centrifuged at max speed ($\sim 18,000$ g) for 20 min at 4 °C. The supernatant was discarded and the resulting pellet was washed with 10 \times volume of ice-cold 70% EtOH and centrifuged for 1–3 min at max speed. This step was repeated two more times. After the final wash, the supernatant was discarded and the pellet was air dried for approximately 5 min before it was resuspended in water. When the MEGAscript T7 Transcription Kit was used for IVT, purification was followed by denaturation of the RNA at 65 °C and enzymatic (Cap1) capping of the RNA using the ScriptCap 2'-O-methyltransferase Kit (CellsScript) and ScriptCap m7G Capping System (CellsScript) for 30–60 min at 37 °C. A second purification using either the RNeasy Mini Kit (Qiagen) or lithium chloride precipitation was required before transfection. Unless otherwise stated, transfections were performed with 1 μ g of replicon RNA per sample for BHK-21 cells and 100 ng of replicon RNA for C2C12 cells.

Modified mRNA. Template DNA for IVT was generated by PCR from Level 1 plasmids, using a forward primer containing a T7 promoter sequence and a sequence complementary to the plus side of the SGP and a reverse primer binding the 3' UTR. Following PCR, DpnI (NEB) was added to the reaction to digest template plasmid DNA and PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). IVTs were performed using MegaScript T7 kit (Life Technologies) at 37 °C for 4 h, except the UTP component of the kit was replaced by N¹-methylpseudouridine-5'-triphosphate (TriLink BioTechnologies). After DNase treatment, RNA was purified using either the RNeasy Mini Kit (Qiagen) or lithium chloride precipitation, as previously described. Resulting mRNAs were enzymatically (Cap1) capped using the ScriptCap 2'-O-methyltransferase Kit (CellsScript) and ScriptCap m7G Capping System (CellsScript) for 30–60 min at 37 °C. Capped mRNA transcripts were subsequently polyadenylated using the A-Plus Poly(A)-Tailing Kit (CellsScript) for 30 min at 37 °C according to the manufacturer's protocol. A second purification was performed before transfection.

Cell culture and transfection. RNA transfections were conducted either in BHK-21 cells as previously described³¹ or C2C12 mouse myoblasts. BHK-21 cells were cultured in EMEM (ATCC) while C2C12 cells were cultured in DMEM (ATCC). Both were supplemented with 10% Tetracycline Screened HyClone FBS (GE Healthcare) at 37 °C and 5% CO₂. Cells at approximately 70% confluence were electroporated using the Neon Transfection System (Life Technologies) following cell line optimization, according to the manufacturer's instructions. Optimal electroporation conditions were 1,100 mV, 40 ms, and 1 pulse for BHK-21 cells and 1,400 mV, 20 ms, and 1 pulse for C2C12 cells. In general, for a 10 μ l transfection into a single well of a 24-well plate (Corning), approximately 100,000 BHK-21 cells or 50,000 C2C12 cells were electroporated. 1 μ g of RNA was transfected per 100,000 BHK-21 cells, and 100 ng of RNA was transfected per 50,000 C2C12 cells unless otherwise stated. Experiments were performed in either 24- or 96-well plates (Corning) with the plating density scaled accordingly. For experiments incorporating small-molecule regulation, 1,000 \times stock solutions of 10 mM TMP in DMSO and 1 mg/ml Dox in water were added for final concentrations of 10 μ M and 1 μ g/ml, respectively.

Flow cytometry. Cells were washed with 1 \times PBS (Corning), trypsinized (Corning), quenched with cell growth media, and resuspended in 1 \times PBS. Flow cytometry was performed using a BD LSRFortessa Flow Cytometer System (BD Biosciences), equipped with 405, 488, and 561 nm lasers. FACSDiva software was used for initial data collection. We collected 10,000–30,000 events per sample for 24-well plates and 2,000–10,000 events per sample for 96-well plates. Technical duplicates were run in addition to experimental replicates for experiments run using 96-well plates. Fluorescence data was acquired with the following cytometer settings: 488 nm laser and 530/30 nm band-pass filter for mVenus, 561 nm laser and 610/20 nm filter for mKate, and 405 nm laser and 450/50 filter for EBFP2. Data analysis was performed with FlowJo V10. For all fluorescence assays, populations of viable, single cells were determined based on forward and side scatter as shown in Supplementary Fig. 29a. Replicon and modRNA electroporation typically exceeded 90% transfection efficiency, and therefore all single cells were taken into account for calculating arithmetic or geometric mean fluorescence. However, high levels of L7Ae caused toxicity and cell death when driven from replicons. Thus, for replicon switches that used Dd-L7Ae, when L7Ae was stabilized using TMP, we observed a significant increase in the number of nonfluorescent cells, as shown in Supplementary Fig. 29b. Presumably, cells with high levels of stabilized L7Ae grew more slowly or died, allowing a larger nontransfected population. This effect could not be accounted for using the single reporter replicon OFF and ON switches, as there was no transfection marker to differentiate nontransfected cells from cells in the "OFF"

state. However, using the two-output switch, we were able to gate cells expressing either mKate or mVenus, removing the negative population to observe only transfected cells.

Luciferase measurement. Cells were prepared using the Steady-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol. Briefly, 100 μ l of Steady-Glo Reagent, equal to the culture volume, was added to each well containing cells in a 96-well plate. After a 5-min incubation, the lysed cells were transferred to black 96-well glass-bottom plates (Corning) and luminescence was read using the Tecan Safire 2.

RNA extraction and qPCR. RNA from cells transfected with two-SGP replicons constitutively expressing mVenus and mKate was extracted 24 h post-transfection using the Direct-zol RNA Miniprep Kit (Zymo Research). 500 ng of RNA was reverse transcribed using a poly(T) primer with the SuperScript III Reverse Transcription Kit (Invitrogen). Resulting cDNA was subjected to RT-PCR using the KAPA SYBR FAST Universal 2 \times qPCR Master Mix (Kapa Biosystems). RT-PCR was performed on a Mastercycler ep Realplex (Eppendorf). Primers were designed to bind within the 5' end of nsP4, the middle of mVenus, and the 3' end of mKate. The relative amounts of each were normalized to GAPDH on a per sample basis. Here, the amounts of mVenus and mKate included both the genomic and subgenomic RNA. The amount of nsP4 was subtracted from these values to determine the relative amount of subgenomic RNA for either mVenus or mKate compared to GAPDH.

Primer sequences. mVenus-qPCR-F GCACAAGCTGGAGTACAACCTAC

mVenus-qPCR-R GATGTTGTGGCGGATCTTGAA
mKate-qPCR-F GATCCAAGAAACCCGCTAAGAA
mKate-qPCR-R CTGCTCGACGTATGCTCTTTG
nsP4-qPCR-F GTGTTGGAGAGACCGAATTG
nsP4-qPCR-R CTGTTAGCAGGTGTGGGATTT
GAPDH-qPCR-F GAACGGGAAGCTTGTCACTA
GAPDH-qPCR-F GCCAGTAGACTCCACAACATAC

Additional sequences. *Replicon sequence.* 5' UTR | nsP1 | nsP2 | nsP3 | nsP4 | subgenomic 5' UTR | mVenus | truncated E1 | 3' UTR | polyA.

5' UTR. ATGGGCGGCGCATGAGAGAAGCCAGACCAATACCTACCCAAA

nsP1. ATGGAGAAAGTTACAGTGTGACATCGAGGAAGACAGCCCATTCCT
CAGAGCTTTGACGCGGACTTCCGCGAGTTTGTAGGTAGAAGCCAAGCAG
GTCACGTATAATGACCATGCTAATGCCAGAGCGTTTTCGCATCTGGCTTC
AAAACGTGATCGAAACGGAGGTGGACCCATCGACACGATCCTTGACATT
GGAAGTGCGCCGCCCGCAGAATGTATTCTAAGCAACAAGTATCATTTGTA
TCTGTCCGATGAGATGTGCGGAAGATCCGGACAGATTGTATAAGT
ATGCAACTAAGCTGAAGAAAAGCTGTAAGGAAATAACTGATAAGGAAT
TGGACAAGAAAATGAAGGAGCTCGCCGCCGTCATGAGCGACCCCTG
ACCTGGAAGCTGAGACTATGTGCTCCACGACGACGAGTCTGTGTC
GCTACGAAGGGCAAGTGTCTGTTTACCAGGATGTATACCGGGTTG
ACGGACCGACAAGTCTCTATACCAAGCCAATAAGGGAGTTAGAGT
CGCCTACTGGATAGGCTTTGACACCCTCTTTATGTTTAAAGAA
CTTGGCTGGAGCATATCCATCATCTACCAACTGGCCGAC
GAAACCGTGTAAACGGCTCGTAACATAGGCCTATGACGCTCTGACGTTAT
GGAGCGGTACGTTAGAGGATGTCCATTCTTAGAAAGAAAGTAT
TTGAAACCATCCAACAATTTCTATTTCTGTTGGCTCGACCATCT
ACCAGAGAAGAGGGAAGTACTGAGGAGCTGGCACCTGCCGCTCTG
TATTTCACTTACGTGGCAAGCAAAATACACATGTCCGTTGTGAGACTATA
GTTAGTTGGGACGGGTACGTCGTTAAAAGAAATAGCTATCAGTCCAGGC
CTGTATGGGAAGCCTTCAGGCTATGCTGCTACGATGCACCGCGAGGG
ATTCCTGTGCTGCAAAAGTACAGACACATTTGAACGGGGAGAGGGTCTCT
TTTCCGCTGTGACGATGTGCCAGCTACATTTGTGTGACCAAATGACTG
GCATAGTGGCAACAGATGTGAGTGGGACGACGCGCAAAAAGTCTGGT
TGGCTCAACAGCGTATAGTCTGCAACGGTTCGACCCAGAGAACA
CCAATACCATGAAAAATTACCTTTTGGCCGTAGTGGCCAGGCA
TTTGCTAGGTGGGCAAGGAATATAAGGAAGATCAAGAAGATGAAAGGC
CACTAGACTACGAGATAGACAGTTAGTCACTATGGGGTGTGTTGTGGG
CTTTTAGAAGGCACAAGACATCTAATTTATAAGCCGCGGATAC
CAAACCATCATCAAAGTGAACAGCGATTTCCACTCATTCTGTGCTG
CCCAGGATAGCGAGTAACACATTTGGAGATCGGGGTGAGAACAAGAA
TCAGGAAAATGTTAGAGGAGCACAAGGAGCCGTCACCTCTCAT
TACCCTGAGGACGTACAAGAGCTAAGTGCAGCGAGCGATGAGG
CTAAGGAGGTGCGTGAAGCCGAGGAGTTGCGCGCAGCTCTAC
CACCTTTGGCAGCTGATGTTGAGGAGCCCACTCTGGAAGCCGAT
GTCGACTTGATGTTACAAGAGGCTGGGGCC

nsP2. GGCTCAGTGGAGACACCTCGTGGCTTGATAAAGGTTACCAGCTA
CGATGGCGAGGACAAGATCGGCTCTTACGCTGTGCTTTCTCCGAGC
GTGTACTCAAGAGTGAATAATTTCTTGCATGCCACCTCTCGTGAAC
AAGTCATAGTGATAACACTCTGGCCGAAAAGGGCGTTATGCC

TGGAACCATACCATGGTAAAGTAGTGGTGCCAGAGGGACATGCAA
TACCCGTCCAGGACTTTCAAGCTCTGAGTGAAAGTGCCACCAT
TGTTGACAACGAACGCTGAGTTTCGTAAACAGTACTGCCACCAT
TGCCACATGGAGGAGCGCTGAACACTGATGAGAATATTACAA
ACTGTCAAGCCCAGCGAGCACGCGGCAATACCTGTACGACAT
CGACAGGAAACAGTGCCTCAAGAAAGAACTAGTCACTGGGCTAGGGCTC
ACAGGCGAGCTGGTGGATCCTCCCTTCCATGAATTCGCCCTACGAG
AGTCTGAGAACACGACCAGCCGCTCTTACCAAGTACCAACATAG
GGGTGATGGCGTGCCAGGATCAGGCAAGTCTGGCATCATTAAAAGC
GCAGTCACCAAAAAGATCTAGTGGTGAGCGCAAGAAAGAAAAC
TGTGCAGAAATATAAGGGACGTCAGAAAATGAAAGGGGTGGAGC
TCAATGCCAGAAGCTGTGGACTCAGTGCCTTGAATGGATGCAA
ACACCCCGTAGAGACCCTGTATATTGACGAAGCTTTTGCTTGTGAT
GCAGGTACTCTCAGAGCGCTCATAGCCATTATAAGACCTAAAAAG
GCAGTGTCTGCGGGGATCCCAACAGTGGCTTTTTCATACAT
GATGTGCCTGAAAAGTGCATTTTAAACCAGATTTTGCACACAAGT
CTTCCAAAAAGCATCTCTCGCCGTGACTAAATCTGTGAC
TTCGGTCTGCTCAACCTTGTTTTACGACAAAAAATGAGAACGAGC
AATCCGAAAGACTAAGATTGTGTGACTGACGACCCACTTACCAA
CCTAAGCAGGACGATCTCATTCTCACTTGTTCAGAGGGTGGG
TGAAGCAGTTGCAAAATAGATTACAAAGGCAACGAAATAATGACGGC
AGCTGCCTCTCAAGGGGTGACCCGTAAGGTTGTGTATGCCG
TTCGGTACAAGGTGAATGAAAATCCTCTGTACGACCCACTCAGAACA
TGTGAACGCTCTACTGACCCGACGAGGACCGCATCGTGTG
GAAAACACTAGCCGGCGACCCATGGATAAAAACACTGACTGCC
AAGTACCCTGGGAATTTCACTGCCAGTATAGGAGTGGCAAGC
AGAGCATGATGCATCATGAGGCACATCTGGAGGACCCGAGC
CTACCGACGCTTCCAGAATAAGGCAACGCTGTGTTGGGCCAAGGCTTT
AGTGCCTGTGTAAGACCGCTGGATAGACATGACCACTGACATGAA
AATGGAACACTGTGGATTATTTTGAACCGGACAAAGCTCACTCAG
CAGAGATAGTATTGAACCAACTATGCGTGAGGTTCTTTGGACTCGATCTG
GACTCCGGTCTATTTCTGACCCACTGTTCGGTTATCCATTAGGAATAA
TCACTGGGATAACTCCCGCTCGCTAACATGTACGGGCTGAATAA
AGAAGTGGTCCGTCAGCTCTCTCGCAGGTACCCACAACACTGCTC
GGCAGTTGCCACTGGAAGAGTCTATGACATGAACACTGGTACA
CTGCGCAATATGATCCGCGCATAAACCCTAGTACCTGTAAACAGAA
GACTGCCTCATGCTTTAGTCTCCACCATAATGAACACCACAGAG
TGACTTTCTTCTATTCTGTCAGAAATTTGAAGGGCAGAAGCTGT
CCTGGTGGTGGGGAAAAGTTGTCCGTCACAGGCAAAATGGTTGAC
TGGTTGTGACAGCCGGCTGAGGCTACCTTACAGAGTCTGGTGTGA
TTTAGGCAACCCAGGTGATGTGCCCCAAGATACATAAATTTGTTAATG
TGAGGACCCCATATAAATACCATCACTATCAGCAGTGTGAAGACCA
TGCCATTAAGCTTAGCATGTTGACCAAGAAAGCTTGTCTGCATCTGA
ATCCCGCGGAAACCTGTGTGACGATAGTGTATGGTTACGCTGA
CAGGCGCCAGCAAGGATCATTTGTTGTGATGACCCAGCTGCTTCA
AGTTTTCCCGGGTATGCAACCGAAATCCTCACTTGAAGAGAGC
GAAGTCTGTTTGTATTCTATGGGTACGATGCAAGGCCCGTACGCA
CAATCCTTACAAGCTTTCATCAACCTTGACCAACATTTATACAGGTT
CCAGCTTCCAGCAAGCC GGATGT

nsP3. GCACCTCATATCATGTGGTGGGAGGGGATATTGCCAGGCCACC
GAAGGAGTATTATAAATGCTGCTAACAGCAAAGGACAACCTGGCGGAG
GGGTGTGCGGAGCGCTGTATAAGAAATTCGCCGAAAGCTTCGATT
TACAGCCGATCGAAGTAGGAAAAGCGCGACTGGTCAAAGGTGCGAGCTA
AACATATCATTTACGCTGAGGACCAAACTCAACAAAGTTTCGGA
GGTTGAAGGTGACAAAACAGTTGGCAGAGGCTTATGAGTCCATCGCTAA
GATTGTCAACGATAACAATTAACAAGTCAAGTACGATTTCCACTGTTGTC
CAGCGGCATCTTTCCGGGAACAAGATCGACTAACCCAAATCA
TTGAACCATTTGCTGACAGCTTTAGACACCCTGATGAGCATGTAGCC
ATATACTGCAGGGACAAGAAATGGGAAATGACTCTCAAGGAAGCA
GTGGTATAGGAGAGAAGCAGTGGAGGAGATATGCATATCCGAGGACT
TTCAGTGACAAACCTGATGACAGAGCTGGTGGGGTGCATCCGAAAG
AGTTCTTTGGCTGGAAGGAAGGGTCAAGACAGCAGCATGCGCAAAACT
TTCTCATATTTGGAAGGGACCAAGTTTACCAGGCGGCCAAGGATATAG
CAGAAATTAATGCCATGTGGCCCGTTCACACGGAGGCCAATGAGCAGG
TATGCATGTATCTCTCGGAGAAAGCATGACGAGTATTAGGTCGA
AATGCCCTGCGAAGAGTCCGGAAGGCTCCACAGCCAGCCAGCT
GCCTTGTCTGTGATCCATGCCATGACTCCAGAAAGAGTACAGCGCCT
AAAAGCCTCAGTCCAGAAACAAATACTGTGTGCTCATCTTTCCATTCG
CGAAGTATAAGAACTACTGGTGTGCAAGAAATGCTCCAGCCCTATA
TTGTTCTACCGAAAGTGCCTGGTATATTCCTCAAGGAAGTATCTC
GTGGAACACCACCGGTGACGAGACTCCGGAGCCATCGGCAG
AGAACCAATCCACAGAGGGGACACTGAACAACCCACTTA
TAACCGAGGATGAGACCAGGACTAGAACAACCTGAGCCGATCAT
CATCGAAGAGGAAGAAGAGGATAGCATAAGTTTGTGTCAGATGG
CCCAGCCACAGGTTGCTGCAAGTCCAGGACAGCATTCACGGGCGGCC
TCTGTATCTAGCTCATCTGCTCCATCTCATGATCCGACTTTGAT
GTGACAGTTTATCCACTACTGACCCCTGGAGGGAGTACGCTGA
CCAGCGGGCAACGTCAGCCGAGACTAACTTCTACTTCGCAAAGAGTAT

GGAGTTTCTGCGCGACCGGTGCGCTGCGCCTCGAACAGTATTACAG
 GAACCTCCACATCCCCTCCGCGCACAAGAACCCGTCCTTGCACC
 CAGCAGGGCCTGCTCGAGAACAGCCTAGTTTCCACCCCGCCAGGC
 GTGAATAGGGTGACTAGTAGAGAGAGCTCGAGGCGCTTACCCCGTC
 ACGCACTCCTAGCAGGTCTGCGAGAACCCAGCCTGGTCTCCAACCCG
 CCAGGCGTAAATAGGGTGATTACAAGAGAGGATTTGAGGCGTTCGT
 AGCACAACAACATGACGGTTTGTATGCGGGTGA

nsp4. TACATCTTTCTCCGACACCGGTCAAGGCAATTACAACAAAA
 ATCAGTAAGCAAACCGTGTATCCGAAGTGGTGTGGAGAGGACCGA
 ATTTGGAGATTTCTGATGCCCCGCGCCTCGACCAAGAAAAAGAAGA
 ATTACTACGCAAGAAATTACAGTTAAATCCCACACCTGCTAACAGAA
 CAGATACCAGTCCAGGAAGGTGGAGAACATGAAAGCCATAACAGCTA
 GACGTATTCTGCAAGGCTTAGGCGATTAATTGAAGGCAGAAAGAAAA
 GTGGAGTGTACCGAACCTGCATCTGTTCTTTGATTTTCATCTAGTG
 TGAACCGTGCCTTTTCAAGCCCAAGTCCGAGTGGAAAGCCTGTAA
 GCCATGTTGAAAGAGAATTTCCGACTGTGGCTTCTTACTGTA
 TTATCCAGAGTACGATGCCTATTTGGACATGGTTGACGGAGCTT
 CATGTGCTTAGACACTGCCAGTTTGGCCCTGCAAAGCTGCGCAG
 CTTTCCA AAGAAACACTCCTATTTGGAACCCACAATACGATCGGCAGTG
 CCTTCAGCGATCCAGAACACGCTCCAGAACGTCCTGGCAGCTGCCACA
 AAAAGAAATTCGAATGTCACGCAAAATGAGAGAATTTGCCCGTATT
 GGATTCGGCGCCTTTAATGTGGAATGCTTCAAGAAATATCGGTGTA
 ATAATGAATATTGGGAAACGTTTAAAGAAAACCCATCAGGCTTACT
 GAAGAAAACGTTGTAATTTACATTAACAAATTTAAAGGACCAAAAGCT
 GCTGCTCTTTTTCGGAAGACACATAATTTGAATATGTTGCAGGAC
 ATACCAATGGACAGGTTTGAATGGACTTAAAGAGAGACGTTGAA
 AGTGACTCCAGGAACAAAACATACTGAAGAACGGCCAAAGGTACAGGT
 GATCCAGGCTGCCGATCCGCTGACCAACCGGATCTGTGCGGA
 ATCCACCGAGAGCTGTTAGGATTAATGCGGTCTGCTGCT
 CCGAACATTACACTGTTTGTATGTCGGCTGAAGACTTTGACGCTA
 TT ATAGCCGAGCACTTCCAGCCTGGGGATTGTGTTCTGGAAACTGACAT
 CGCGTCTGTTGATAAAAGTGAGGACGACGCCATGGCTCTGACCCGG
 TTAATGATTCGGAAGACTTAGGTGTGGACGACGAGCTGTTGACGC
 TGATTGAGGCGGCTTTTCGGGCAAAATTTCAATCAATACATTTGC
 CCACTAAAACATAAATTTAAATTCGGAGCCATGATGAAATCTGGAATGT
 CCTCACACTGTTTGTGAACACAGTCATTAACATTTGAATCGCAAGCA
 GAGTCTTGAGAGAACCGCTTAAACCGGATCCACATGTGACGACT
 CATTGGAGATGACAATATCGTGAAGGAGTCAAATCGGACAAATTAAT
 GGCAGACAGGTGCCACCTGTTGAATATGGAAGTCAAAGATTA
 TAGATGCTGTGGTGGGAGAAAGCGCTTATTCTGTGGAGGTTTATT
 TTGTGTGACTCCGTGACCGGCACAGCGTGGCTGTGGCAGACCCCTAA
 AAAGGCTGTTTAAAGCTTGGCAAACCTCTGGCAGCAGACGATGAA
 CATGATGATGACAGGAGAAGGCAATGTCATGAAGAGTCAACACGCG
 TGAACCGAGTGGGTTCTTTCAAGAGCTGTGCAAGGCAAGTGA
 ATCAAGGATGAAACCGTAGGAACTTCCATCATAGTTATGGCCAT
 GACTACTAGCTAGCAGTGTAAATCATTAGCTACTGAGAGGGGCC
 CCTATACTCTCTACGGCTAA

Subgenomic 5' UTR.

CCTGAATGGACTACGACATAGTCTAGTCCGCCAAGGCCACC

mVenus.

ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCATCTGT
 GTGAGCTGGACGGCGACGTAAACGGCCACAAGTTACAGCGTGTCCG
 GCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCTTGAAGCT
 GATCTGCACACCGGCAAGCTGCCGCTGCCCTGGCCACCTCTGTGA
 CCACCTGGGCTACGGCTGCAGTGCTTCGCCGCTACCCCGACCAC
 ATGAAGCAGCAGACTTCTTCAAGTCCGCCATGCCGAAGGCTACGTC
 CAGGAGCGCACCATCTTTCAAGGACGACGGCAACTACAAGACCCGCG
 CCGAGGTGAAGTTCGAGGGCGACACCTGGTGAACCGCATCGAGC
 TGAAGGCGATCGACTTCAAGGAGGACGGCAACATCTGGGGCACA
 AGCTGGAGTACAACACTACAAGCCACAACGCTTATATACCCGCCGACAA
 GCAGAAAGAACGGCATCAAGGCCAATTTCAAGATCCGCGCAACAATCG
 AGGACGGCGGCTGCAGCTCGCCGACCACTACCAGCAGAACACCC
 CCATCGGCGACGGCCCGTGTGCTGCGCCGACAACCACTACCTGAGCTA
 CCAGTCCAAGCTGAGCAAAGCCCAACGAGAAGCGCATCACATG
 GTCCTGTGGAGTTCGTG ACCGCCCGGGATCACTCT
 CGGCATGGACGAGCTGTACAAGTAATAA (double STOP for cloning)

Truncated E1. TATGTTACGTGCAAAGGTGATTGTCACCCCCGAAAGAC
 CATATTTGACACACCCTCAGTATCACGCCAAACATTTACAGCCGCGG
 TG TCAAAAACCGCGTGGACGTGTTAAACATCCCTGCTGGGAGGATCA
 GCCGTAATTTATAATTTGGCTTGGTGTGGCTACTATTGTGGCCATGTA
 CGTGTGACCAACCAGAAACATAATTGA

3' UTR. ATACAGCAGCAATTGGCAAGCTGCTTACATAGAATCTCGGGCGAT
 TGCCATGCCGCTTAAATTTTATTTATTTCTTTCTTTCCGAATC
 GGATTTTGTTTTAAATTTTC

Poly(40)A. AA

Additional elements. 2× K-turn.

GGCGGTGATCCGAAAGGTGACCCGGATCTGGGGCGTGATCCGAAAGG
 TGACCCGGATCCACCGGTC

2× Tet-aptamer.

ATCCAGGCAGAAAGGTGATACGGACGGAATGTGGTGGCCTGGATCA
 ACAACAACAAAATCCAGGCAGAGAAAGGTGATACGGACGGAATGTGGT
 GGCCTGGATCAACAACAACACTGTAA (STOP added to prevent
 undesired protein products)

Ddd sequence.

ATGATCAGTCTGATTTGCGGCTTAGCGGTAGATTACGTTATCGGCATGGA
 AACGCCATGCCGTGGAACCTGCCGATCTCGCCTGGTTAAACGC
 AACACCTTAAATAAACCCGTGATTATGGCCGCCATACCTGGGAATCAA
 TCGGTCTGCTCGTTGCCAGGACGCAAAAATATTATCCTCAGCAGTCAACCG
 AGTACGGACGATCGCGTAACGTGGGTGAAGTGGTGGATGAAGCCATCG
 CGGCGTGTGGTGCAGTACCAGAAATCATGGTGGATTGGCGCGGCTG
 CGTTATTGAACAGTTCTTGCACAAAAGCGCAAAAAGTGTATCTGACGCA
 TATCGACGCAAGGTGGAAGGCGACACCCATTCCCGGATTACGAGC
 CGGATGACT GGAATCGGTATTACGCAATTCACGATGCTGATG
 CGCAGAACTCTCA CAGCTATTGCTTTGAGATTCTGGAGCGGCGA

L7Ae.

ATGTACGTGAGATTTGAGGTTCTGAGGACATGCAGAACGAAGCTCTGA
 GCTGTCTGGAAGAAGTTAGGGAGAGCGGTAAGGTAAGAAAGGTA
 CCAACGAGACGACAAAGGCTGTGGAGAGGGGACTGGCAAAGC
 TCGTTTACATCGCAGAGGATGTTGACCCGCTGAGATCGTTGCTC
 ATCTGCCCTCTCTGCGAGGAGAAGAAATGTCGGTACATTTACGTTA
 AAAGCAAGAACGACCTTGGAAAGGCTGTGGCATTGAGGTGCCATG
 CGCTTCGGCAGCGATAATCA ACGAGGGAGAGCTGAGAAAGGAGC
 TTGGAAGCCTTGTGGAGAAGATTA AGGCCTTCAGAAGTAA

TetR. ATGTCAAGACTCGACAAGAGCAAGGTGATTAACAGTGCCTGG
 AACTTCTCAATGAAGTTGGGATCGAGGGGCTGACTACTAGAAAACCT
 GCACAGAACTGGGGTGTGAGCAGCCACCTGTACTGGCAGGTAA
 AAAAAAGGGCCCTGCTGGATGCTGTGCCATCGAGATGCTGGAT
 AGGCATCATACCCACTTCTGCCCTTGGAAAGGAGATCCTGGCAGGATTT
 CCTAGAAACAACGCCAAGTCTTTCGCTGTGCCCTTCTTAGCCACC
 GGGATGGTGTAAAGTCCATCTCGGCACACACCAACTGAGAAGCAG
 TACGAAACTCTCGAGAACCAGCTGGCCTTCTCTGTCAACAGGGCTT
 TTCTTGTAAAACGCCCTGTACGCACTGAGTGCAGTGGGCACTT
 TACACTCGGATGTTTCTGGAGGACCAAGAATCAGGTGGCAAAG
 GAAGAGAGGGAGACACCTACGACTGACTCCATGCCCTCTCTGTG
 AGGCAGGCAATAGAATGTTTCGACCATCAGGGCGCAGAACCCGCTT
 TCTGTTTGGCTGGAAGTATATCTGCGGCTTGGAAAACAGCTGAAG
 TCGGAGTCCGGAGCTAA

PEST. AAGCTTAGCCATGGCTTCCCGCCGGAGGTGGAGGAGCAGGATG
 ATGGCAC GCTGCCATGTCTTGTGCCAGGAGCGGGATGAGCCGT
 CACCCTGCAG CTTGTGCTTCTGCTAGGATCAATGTGTA

P2A. GGAAGCGGAGCTACTAATTCAGCCTGCTGAAGCAGGCTGGAGACG
 TGGAGGAGAACCCTGGACCT

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that data supporting the finding of this study are available within the article and its Supplementary Information. Sample analysis of cytometry data can be found in Supplementary Fig. 29. Replicon MoClo assembly plasmids have been submitted to Addgene with the accession numbers 115928, 115929, 115930, 115931, 115932, 115933, 115934, 115935, 115936, 115937, 115938, 115939, 115940, 115941, 115942, 115943, 115944, 115945, 115946, 115947, 115948, 115949, 115950, 115951, 115952, 115953, 115954, 115955, 115956, 115957, 115958, 115959, 115960, 115961, 115962, 115963, 115964, 115965, 115966, and 115967. A more detailed table can be found in Supplementary Fig. 30. Additional data are available from the corresponding authors upon reasonable request.

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- Engler, C., Gruetzner, R., Kandzia, R. & Marillonnet, S. Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. *PLoS One* **4**, e5553 (2009).
- Wielgosz, M. M., Raju, R. & Huang, H. V. Sequence requirements for Sindbis virus subgenomic mRNA promoter function in cultured cells. *J. Virol.* **75**, 3509–3519 (2001).

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▶ Experimental design

1. Sample size

Describe how sample size was determined.

A sample size of $n = 2$ per experiment group was chosen to be cost efficient. To guarantee reproducibility, results were successfully reproduced by two or more co-authors of the study.

2. Data exclusions

Describe any data exclusions.

No data were excluded from the study.

3. Replication

Describe the measures taken to verify the reproducibility of the experimental findings.

All experiments have been successfully reproduced by two or more co-authors of the paper.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Cultured cells were randomly allocated into experimental groups by pipetting vortexed samples.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Investigators were not blinded to group allocations. Blinding was not necessary as protein expression levels measured using instruments were not expected to be affected by investigator bias.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- | n/a | Confirmed |
|-------------------------------------|--|
| <input type="checkbox"/> | <input checked="" type="checkbox"/> The <u>exact sample size</u> (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> A statement indicating how many times each experiment was replicated |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> The statistical test(s) used and whether they are one- or two-sided
<i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i> |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Test values indicating whether an effect is present
<i>Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.</i> |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> A clear description of statistics including <u>central tendency</u> (e.g. median, mean) and <u>variation</u> (e.g. standard deviation, interquartile range) |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> Clearly defined error bars in <u>all</u> relevant figure captions (with explicit mention of central tendency and variation) |

See the web collection on [statistics for biologists](#) for further resources and guidance.

► Software

Policy information about [availability of computer code](#)

7. Software

Describe the software used to analyze the data in this study.

MATLAB, Flowjo, and Excel were used to analyze the data in this study.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* [guidance for providing algorithms and software for publication](#) provides further information on this topic.

► Materials and reagents

Policy information about [availability of materials](#)

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

Materials will be available from the research authors or through a third party.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No antibodies were used in this study.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

BHK-21 cells used in this study were described in Beal, J. et al. Model-Driven Engineering of Gene Expression from RNA Replicons. *ACS Synth. Biol.* 4, 48–56 (2015).

b. Describe the method of cell line authentication used.

The BHK-21 cells were not authenticated.

c. Report whether the cell lines were tested for mycoplasma contamination.

The cell lines were tested for mycoplasma contamination. The cells were NOT contaminated with mycoplasma.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

None of the cell lines used are listed in the ICLAC database.

► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

No research animals were used in this study.

Policy information about [studies involving human research participants](#)

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Humans were not used as research subjects in this study.