Molecular Therapy
Methods & Clinical Development
Original Article

All-in-one IQ toggle switches with high versatilities for fine-tuning of transgene expression in mammalian cells and tissues

Jeongkwan Hong,[1,6] Kyung-Cheol Sohn,[2,6] Hye-Won Park,[3,6] Hyoeun Jeon,[3] Eunjin Ju,[2] Jae-Geun Lee,[4] Jeong-Soo Lee,[4,5] Jaerang Rho,[3] Gang Min Hur,[2] and Hyunju Ro[1]

[1]Department of Biological Sciences, College of Bioscience and Biotechnology, Chungnam National University, Daejeon 34134, Korea (ROK); [2]Department of Pharmacology, College of Medicine, Chungnam National University, Daejeon 301 747, Korea (ROK); [3]Department of Microbiology and Molecular Biology, College of Bioscience and Biotechnology, Chungnam National University, Daejeon 34134, Korea (ROK); [4]Microbiome Convergence Research Center, KRIBB, 125 Gwahak-ro, Yuseong-gu, Daejeon 34141, Republic of Korea; [5]KRIBB School, University of Science and Technology, 125 Gwahak-ro, Yuseong-gu, Daejeon 34141, Republic of Korea

The transgene toggling device is recognized as a powerful tool for gene- and cell-based biological research and precision medicine. However, many of these devices often operate in binary mode, exhibit unacceptable leakiness, suffer from transgene silencing, show cytotoxicity, and have low potency. Here, we present a novel transgene switch, SIQ, wherein all the elements for gene toggling are packed into a single vector. SIQ has superior potency in inducing transgene expression in response to tebufenozide compared with the Gal4/UAS system, while completely avoiding transgene leakiness. Additionally, the ease and versatility of SIQ make it possible with a single construct to perform transient transfection, establish stable cell lines by targeting a predetermined genomic locus, and simultaneously produce adenovirus for transduction into cells and mammalian tissues. Furthermore, we integrated a cumate switch into SIQ, called SIQmate, to operate a Boolean AND logic gate, enabling swift toggling-off of the transgene after the removal of chemical inducers, tebufenozide and cumate. Both SIQ and SIQmate offer precise transgene toggling, making them adjustable for various researches, including synthetic biology, genome engineering, and therapeutics.

INTRODUCTION

Tight manipulation of transgene induction, governed by a sophisticated engineered promoter with regulatable enhancer elements, has shed light on new avenues for elucidating the physiological function of genes of interest and for clinical applications of gene therapy. Since the application of the lac operator-repressor[1] and tetracycline (Tet)-responsive transgene activation system[2] on mammalian cells, tremendous efforts have been devoted to developing far more reliable toggle gene switches applicable not only at the cellular level, but also at the whole organism level.[3–7] Thus far, developed transgene switches exploit tunable external stimuli, for instance, discrete hormones, chemicals, heavy metals, or physical stresses such as heat, light, magnetic fields, ultrasonic sound, and electrical signals.[8,9] Among the aforementioned stimuli, chemically based systems have been highly sought because of the convenience of manipulating transgene induction levels via simple dosing with a small molecule at different concentrations and for different time windows.

Among the small molecule-dependent transgene switches, the Tet transactivator (Tet-On/Off) systems, exploiting Tet or its derivatives like doxycycline and minocycline as a triggering cue, and the Gal4/UAS system adopting upstream activation sequence (UAS) and a chimeric protein composed of Gal4 DNA binding domain (DBD), small molecule binding domain (ligand-binding domain), and transactivator domain (AD) responsive to appropriate chemical inducers such as RU486 (mifepristone) or tebufenozide (Teb), have been most widely used to modulate transgene induction and scrutinized for clinical applications.[6,10–14] However, although exploiting the Tet-On/Off system has been considered as the most powerful method to induce transgene expression, the frequently observed unacceptable level of leakiness constrains its broad applications in biological researches, especially when tight control of transgene expression is required. The flaw of the Tet-On/Off system was significantly rectified by consolidating the binary mode of the Tet-On/Off system into a single vector optimized for lentivirus production.[15–18] The all-in-one Tet-Off system decreased basal leakiness, but at the expense of the strength of the transgene induction ability.[15] Despite several improvements applied to lentiviral-based transgene switches, the intrinsic

Received 16 August 2023; accepted 29 January 2024;
https://doi.org/10.1016/j.omtm.2024.101202.

[6]These authors contributed equally

Correspondence: Jaerang Rho, Department of Microbiology and Molecular Biology, College of Bioscience and Biotechnology, Chungnam National University, Daejeon 34134, Korea (ROK).
E-mail: jrrho@cnu.ac.kr
Correspondence: Gang Min Hur, Department of Pharmacology, College of Medicine, Chungnam National University, Daejeon 301 747, Korea (ROK).
E-mail: gmhur@cnu.ac.kr
Correspondence: Hyunju Ro, Department of Biological Sciences, College of Bioscience and Biotechnology, Chungnam National University, Daejeon 34134, Korea (ROK).
E-mail: rohyunju@cnu.ac.kr

propensity of its host genome integration must be considered when applying the lentiviral switch to gene therapy.[18] While nonintegrating lentiviral vectors (NILs) have been developed[19,20] and used for gene editing with a singular mode,[21] chemically elicitable singular transgene switches based on NILs have not been reported yet. Meanwhile, the critical flaw of the Gal4/UAS system is its weaker potency in inducing transgene expression compared with other gene switches, such as the Tet-On/Off system.[14] In a preclinical approach, a Gal4/UAS transgene induction system had been successfully delivered into the rat brain via adeno-associated virus (AAV), which was able to be stimulated by the intraperitoneal injection of RU486.[22] The binary mode of the Gal4/UAS-based AAV transgene switch was ingeniously consolidated into a single AAV vector in the following studies.[23,24] In these studies, regulatable glial cell-derived neurotrophic factor expression in the rodent brain by boosting the function of RU486 has shown therapeutic potential by ameliorating the symptoms of neurodegenerative disorders. Thus, the ideal transgene toggle switch should possess the singular qualities of being non-leaky while maintaining strong transgene induction potency.

A repressible binary transgene induction system, the Q switch, derived from the fungus *Neurospora crassa*,[25] was adopted in various organisms as a novel transgene induction system.[26–33] After extensive modification by several research groups, the Q switch became one of the most attractive transgene switches, with various beneficial features, including exquisite sensitivity, relatively low toxicity, and being free from transgene silencing problems. Recently, we reported a more reliable binary Q switch, IQ-Switch, optimized for the transgenesis of zebrafish.[34] The IQ-Switch showed almost undetectable leakiness of transgene expression, eliciting at least 4.5-fold more sensitivity to a chemical inducer Teb in comparison to Gal4/UAS under identical experimental conditions in zebrafish.[34]

We previously demonstrated that the Gal4/UAS-based singular gene switch (EUI) was successfully implemented for transgene induction in human cell lines and rodent tissues.[14,35] However, there are several issues that need to be critically improved for broader usability in biological research. First, the transgene inducibility of the Gal4/UAS system is generally weaker than that of other widely used gene switches, such as the Tet-On/Off system. Second, the Gal4/UAS system is highly vulnerable to enhancer methylation, which can result in transgene silencing after consecutive generations. Third, since there are two discrete vectors optimized for transient transfection (pEUI(+)) and adenovirus production (pENTR-EUI), respectively, combining the two features into one would be ideal for the ease of conducting experiments. Fourth, establishing stable cell lines using pEUI(+) has not been an easy task because the vector lacks any auxiliary sequences that aid in its genome integration.

Here, we report a novel singular IQ-Switch (henceforth SIQ) equipped with all the necessary components for transgene induction in mammalian cells and rodents' tissue. The SIQ shows none of the leakiness of the transgene expression while maintaining high sensitivity to the chemical inducer. We also included two distinct integrase recognition sites to induce genome integration into a specific locus and to produce adenoviral vectors via recombination events. With a single vector of SIQ, we successfully generated several cell lines with switchable transgenes integrated at an identical genomic locus and efficiently produced adenovirus for the transduction of the same tunable transgenes into cells and tissues that are refractory to conventional transfection approaches. Another advantage of the singular feature of SIQ is that no further molecular cloning is required for adenovirus preparation. In addition, by adapting transcriptional Boolean logic gates, we tested the utility of SIQ for synthetic biology purposes. We showed that the SIQ-based logic circuit combined with a transcriptional repression device guides more swift togging-off of transgene switch. Altogether, the novel SIQ and its derivative enable the researchers to investigate the cellular roles of genes of interest under physiologically relevant conditions with tunability, robustness, versatility and reliability.

RESULTS

The "all-in-one" SIQ is far more potent for transgene induction than Gal4/UAS

To address the flaws of EUI gene switch, we attempted to integrate the binary IQ-Switch, originally optimized for zebrafish transgenesis,[34] with several modifications. To create a novel SIQ, we assembled all the necessary elements for IQ-based transgene toggling into a pENTR-EUI vector. The Gal4-VP16-EcR transactivator of pENTR-EUI was replaced with an IQ-Switch module composed of a QFDBD (which recognizes QUAS), a 2× QF minimal activation domain (2×AD*), a minimal VP16 activation domain (VP16*), and a domain of the ecdysone receptor (EcR), all of which are located upstream of a bGH poly(A) signaling sequence. We substituted 13×QUAS for the 10×UAS of the vector, followed by an SV40 poly(A) signaling sequence. The multiple cloning site (MCS) located immediately after a carp beta-actin minimal promoter consisted of the following restriction enzymes in sequence: BspD I, Cla I, BspE I, Afl II, Not I, and Sca I. To facilitate the establishment of cell lines with genes of interest, we introduced a phiC31 serine integrase recognition site, referred to as attB,[36] downstream of a bGH poly(A) signaling sequence and upstream of the 13×QUAS. Additionally, a puromycin selectable marker, controlled by the SV40 promoter, was inserted between the pUC origin (ori) of replication and the attL1 recombination site. The kanamycin resistance gene remained unaltered. The finalized vector, designated as pSIQ (GenBank accession number: OR355468), was designed to lose both the pUC ori and two selectable markers after recombining with a pAd/PL-DEST (Invitrogen) vector for the preparation of adenovirus (Figures 1A and 1B).

To investigate whether pSIQ could operate as expected in human cell lines, we performed transient transfection in HEK293 cells using pEUI(+) for a direct comparison. Although both plasmids exhibited *EGFP* transgene expression in a Teb dosage-dependent manner, the expression from pSIQ was significantly more potent than that from pEUI(+), even when EGFP expressivity seemed to saturate at doses exceeding 5 μM of Teb (Figure 2A). The enhanced sensitivity of

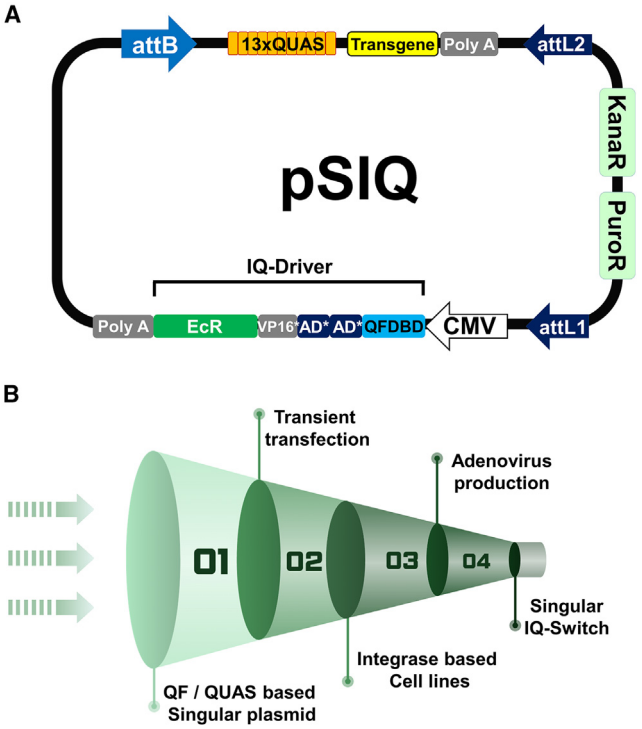


Figure 1. A schematic diagram of SIQ transgene toggle switch

(A) The pSIQ vector encompasses all the components of an IQ-Switch, including an IQ-Driver: QFDBD, two minimal activation domains of QF (2×AD*), a minimal VP16 activation domain (VP16*), and an EcR. These components are governed by a CMV promoter and required for binding to the 13× QUAS enhancer elements, subsequently inducing transcription of genes of interest only when stimulated by Teb. A phiC31 serine integrase recombination site is denoted as attB, where the recombination event occurs to the corresponding attP site in the presence of the recombinase. For ease of cloning into the pAd/PL-DEST destination vector, we included gateway cloning-associated elements (attL1/attL2) for the production of adenovirus. The kanamycin and puromycin antibiotic-resistant markers are denoted as KanaR and PuroR, respectively. (B) The all-in-one SIQ transgene toggle switch exhibits high versatility. With a single construct, a broad range of tailored transgene induction experiments can be carried out through transient transfection, generation of stable cell lines, and adenovirus-mediated transgene delivery. The arrows indicate that discrete purposes of experiments could be merged into a SIQ.

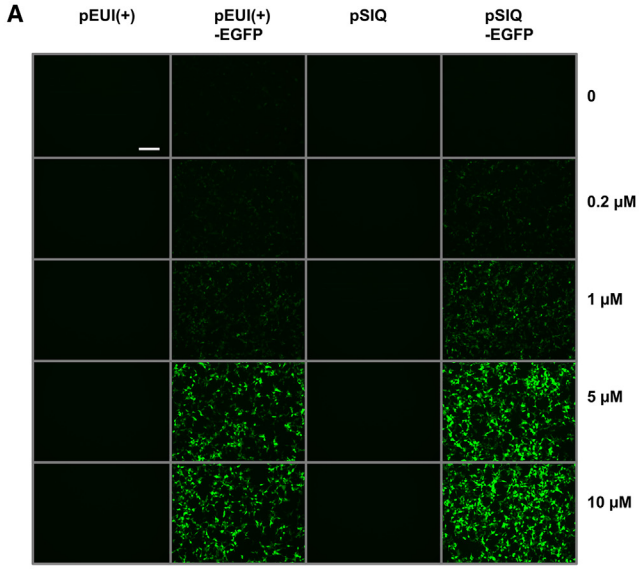
pSIQ compared with pEUI(+) was quantitatively validated using a dual luciferase assay. As shown in Figure 2B, in comparison with the inducibility of the EGFP transgene, both plasmids reached a plateau at 1 μM Teb. Notably, pSIQ stimulated the luciferase reporter significantly more than pEUI(+) did at any Teb dose. Importantly, we observed an approximately 40% lower basal level of leakiness with pSIQ compared with pEUI(+) (Figure 2C). Taken together, our data show that pSIQ is far more potent and reliable than the previously suggested pEUI(+) transgene toggling system.

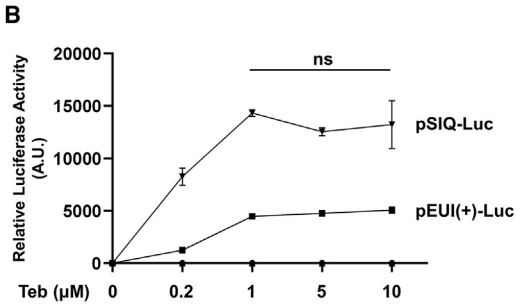
SIQ makes it easy to generate stable cell lines

The establishment of stable cell lines has many advantages, encompassing features for studying the exquisite mechanisms of genes or the development of new therapeutics.[37] However, it is concomitant with laborious procedures. As we mentioned above, the pSIQ equipped with serine integrase recognition sequence (attB) provides a convenient method for generating stable cell lines through integrase-based genome recombination.[36] To facilitate the genome integration of transgenes subcloned in pSIQ, we initially allocated an attP site into the genome of host HEK293 cells by means of a lentiviral transduction system. The commercially available lentiviral vector (pCDH-CMV-MCS-EF1α-Puro, System Biosciences) was subjected to modifications to replace the antibiotic-resistant gene, *puromycin N-acetyltransferase*, with *blasticidin deaminase*, whose expression is governed by an EF1α promoter. Additionally, the CMV7 promoter was removed, and an attP phiC31 recognition site was subcloned into the upstream of EF1α promoter. We used the vector designated as pBla-attP-Lenti for packaging lentivirus to infect HEK293 cells. After subjecting the cells to selective pressure with blasticidin S antibiotics, the surviving cells were individually selected to establish stable clones (HEK293-attP-Bla), each carrying the attP site. We co-transfected the pSIQ encoding an *EGFP* transgene with the phiC31 integrase expression vector in HEK293-attP-Bla. Subsequently, we treated the cells with puromycin to select those resistant to the antibiotic treatment. Successfully integrated clones (SIQ-EGFP) were then validated by genomic PCR (Figures 3A and 3B), confirming their correct implantation into the attP site of the host genome.

To induce EGFP expression, we treated the SIQ-EGFP with Teb. As shown in Figure 3C, the EGFP fluorescence signal was exclusively detected in cells treated with Teb. After Teb stimulation, we observed a rapid increase in EGFP expression, detectable as early as 3 h after treatment (Figure 3D). The expression level of EGFP continued to rise with continuous Teb treatment, reaching a plateau at 48 h (Figure 3D). Importantly, the established cell line responded exclusively to exogenous chemical stimuli, as evidenced by the gradual decrease in the level of *EGFP* transgene as time goes on until 48 h after the removal of Teb (Figure 3E). This ability of SIQ to fine-tune the EGFP expression suggests that the toggle switch could be a valuable tool for various cellular applications. To revalidate the high inducibility of Teb and the low leakiness in the SIQ system, fluorescence-activated cell sorting (FACS) analysis was performed. We aimed to demonstrate the fidelity of SIQ by generating another stable cell line with an *EGFP* transgene and all the SIQ components except for the driver module (CMV-QFDBD-2x AD*-VP16*-EcR-poly(A)). This cell line was targeted to the identical attP locus of HEK293-attP-Bla, enabling a direct comparison with SIQ-EGFP (Figure 3F). As expected, the cell line lacking the driver module exhibited no response to Teb stimuli, while over the 94% of SIQ-EGFP cells were sorted as EGFP positive when stimulated with Teb (Figure 3G). Importantly, the SIQ-EGFP without Teb treatment displayed only a negligible background level of EGFP expression (Figure 3G).

The high fidelity of toggle switches lies in their low or absent transgene leakiness. This characteristic is of critical importance, especially when the transgenes have the potential to be highly toxic to the cells. Continuous leakiness could lead to harmful effects on the host cell's viability and survival. Consequently, in such cases, establishing stable





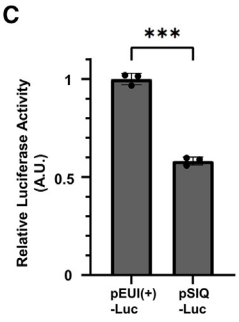


Figure 2. SIQ surpasses the Gal4/UAS-based singular gene switch regarding sensitivity and the issue of leakiness

(A) The pEUI(+)-EGFP refers to a previously reported singular gene switch based on the Gal4/UAS system. The gradually elevated induction of the EGFP reporter, dependent on Teb dosage, was visualized under a fluorescence microscope after transiently transfecting 1 μg of the indicated plasmid constructs. After 24 h of transfection, Teb was treated for 24 h, and then the expression level of EGFP was observed using the same exposure times and contrasts. Scale bar, 200 μm. (B) Quantitative measurements of transgene expression levels were performed using the dual-luciferase assay. HEK293 cells were transfected with either pEUI(+)-Luciferase or pSIQ-Luciferase. After transfection, the cells were treated with Teb for 8 h. Statistical significance was analyzed using two-way ANOVA with Šidák correction (n = 3). ns, not significant. (C) The basal leakiness of pEUI(+) and SIQ was compared through a dual luciferase assay in the absence of Teb. Statistical significance was explored using the Student t test. The data are represented as mean ± SD (n = 3). ***$p < 0.001$. A.U., arbitrary unit; Luc, luciferase.

cell lines would not be practical. To address the issue of transgene leakiness, we exploited a virulent enzyme, nitroreductase, known to induce cellular apoptosis in the presence of specific substrates.[38] Among the several variants of the enzyme, we chose *NTR-2.0* (NfsB_Vv-F70A/F108Y) as a transgene for the readout of the leakiness since the modified nitroreductase has been considered the most sensitive to metronidazole (MTZ), which is innocuous to cells but turns into a highly cytotoxic metabolite in the presence of NTR-2.0.[39] Thus, if the *NTR-2.0* transgene under the control of the SIQ switch were uncontrollably leaky beyond the threshold level, the cells would undergo apoptosis in the presence of MTZ. To measure the level of leakiness, we generated a stable cell line that harbored the *NTR-2.0* transgene governed by the SIQ toggle switch incorporated into the attP locus of HEK293-attP-Bla. The EGFP detection marker was co-expressed with NTR-2.0, linked via P2A, a self-cleaving 2A peptide.[40] As expected, the stable cell line treated with Teb alone showed EGFP fluorescence and did not manifest any morphologically discernible defects (Figures 4A and S1A). Similarly, cells administered with MTZ alone remained healthy even under high concentrations of MTZ (Figure S1B). However, a typical phenomenon of cell death was observed, with cells becoming spherical in shape and then freely floating in the culture media only when both Teb and MTZ were added to the culture condition (Figures 4A and S1). The MTZ-induced cell death triggered by NTR-2.0 was largely inhibited through the addition of carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-fmk), a pan-caspase inhibitor (Figure 4A).[41] The quantitative measurement of caspase 3 and 7 activities reinforced our observation that the SIQ toggle switch was not leaky and was tightly controlled by the input of Teb stimuli. Only when treated with both Teb and MTZ, the cells showed significantly elevated caspase activity (Figure 4B). The potency of pSIQ was compared with the Gal4/UAS transgene switch, pEUI(+),[14] using the MTT assay to evaluate cell viability. The cells transiently transfected with pSIQ-EGFP-P2A-NTR-2.0 became most vulnerable to treatment with MTZ only when co-treated with Teb (Figure S2). Thus, MTZ-dependent cell death resulted from the induction of NTR-2.0 by Teb, with a higher induction level observed in the SIQ switch compared with the Gal4/UAS system.

A SIQ toggle switch can be repurposed as an adenovirus gene delivery system

Adenovirus-mediated gene delivery systems have been extensively used for clinical applications.[42] The viral vector exhibits the ability to infect a wide range of cell lines and tissues.[43] Moreover, the episomal nature of adenovirus ensures that it does not interfere with the integrity of the host cell genome, resulting in a gradual reduction of the viral genome within the cells over time. To facilitate the efficient delivery of SIQ into cells that are highly refractory to accepting exogenous DNA, we

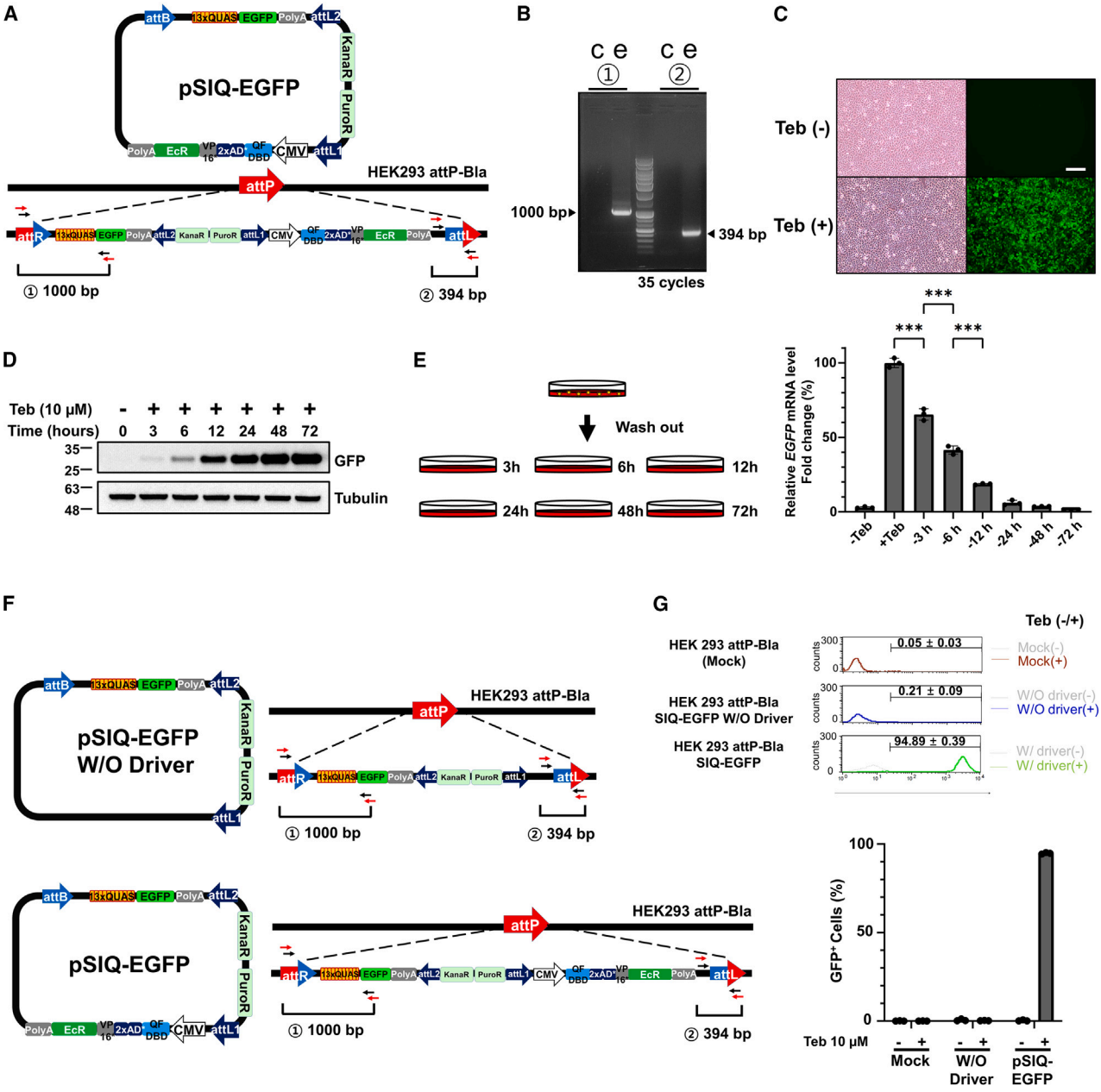


Figure 3. Stable cell line harboring the SIQ system could be generated by integrating it into a specific locus of the host cell genome

(A) A schematic diagram illustrating the phiC31-mediated genomic integration of the SIQ toggle switch. The phiC31 serine integrase targets the attB element of pSIQ and an attP site in the host cell genome, mediating directional recombination, resulting in producing attR and attL recombined sequences. (B) A representative result of genomic PCR using the primers depicted with red and black arrows in (A). The first PCR products amplified by primers labeled with red arrows were used for the second PCR reaction with primers depicted by black arrows. The expected size of PCR products was shown in (A) under the square brackets. c, control group with mock cell genome for PCR template; e, experimental group. (C) Established stable cell lines harboring SIQ-EGFP were exquisitely sensitive to Teb stimuli. EGFP was detected only in the group of cells treated with 10 μM Teb for 24 h. Bright field images were located on the left side. Scale bar, 200 μm. (D) EGFP was detected as early as 3 h after 10 μM of Teb treatment, as shown by western blotting. Endogenous expression of α-tubulin was used as a loading control. (E) After 24 h of Teb (10 μM) treatment, the cells were reseeded into fresh culture dishes to minimize Teb presence. The level of EGFP transcripts was measured by qRT-PCR after harvesting the cells at the indicated time points. The data are presented as mean ± SD. Scale bar, 200 μm. Statistical analysis was performed using One-way ANOVA with Tukey's HSD test (n = 3). ***$p < 0.001$. (F) Schematic comparison between pSIQ-EGFP without IQ-Driver (W/O Driver) and pSIQ-EGFP constructs. (G) FACS analysis of Mock (containing only an attP in their genome), SIQ-EGFP W/O Driver, and SIQ-EGFP stable cell lines after 24-h exposure to Teb (10 μM). Data are presented as mean ± SD (n = 3).

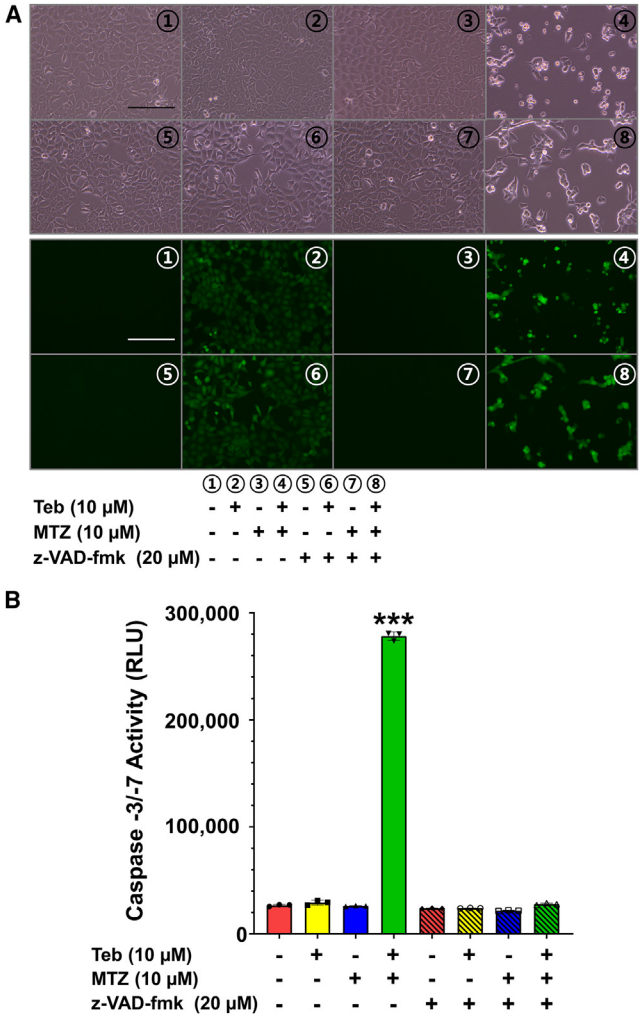


Figure 4. SIQ toggle switch was free from transgene leakiness

(A) Bright field and GFP channel images of an established cell line harboring SIQ-EGFP-P2A-NTR 2.0 at the attP locus of HEK293 cells after 24 h of treatment with the indicated combinations of chemicals. Scale bar, 200 μm. (B) Measurement of caspase activities in the stable cell line harboring SIQ-EGFP-P2A-NTR 2.0 after 24 h of treatment with the indicated chemicals. Statistical analysis was performed using ordinary One-way ANOVA with Tukey's HSD test (n = 3). The data are presented as mean ± SD. ***$p < 0.001$.

adopted the adenovirus packaging system. The pSIQ vector, with *EGFP* as a transgene, was integrated *in vitro* with a pAd/PL-DEST destination vector (Invitrogen) using gateway cloning system (Invitrogen) to produce adenovirus (Ad/SIQ-EGFP). We also prepared a previously reported adenoviral vector (pENTR-EUI), which responds to identical chemical stimuli, such as Teb.[14] The pENTR-EUI, carrying the EGFP reporter, was used for adenovirus production (Ad/EUI-EGFP) after merging it with the pAd/PL-DEST destination vector. Since the expression of *EGFP* transgene in the pENTR-EUI is controlled by the Gal4/UAS toggle switch, it serves as a rational control for comparison with the SIQ system.

Under the conventional transfection approaches, HaCaT keratinocytes are known to exhibit very low transfection efficiency.[44] After infecting HaCaT cells with Ad/SIQ-EGFP and Ad/EUI-EGFP, we treated them with 10 μM Teb for an additional 48 h before analyzing the expression level of EGFP. Either Ad/SIQ-EGFP- or Ad/EUI-EGFP-infected HaCaT cells exhibited a gradual increase in EGFP fluorescence corresponding with the elevated multiplicity of infection (MOI) (Figure 5A). Nevertheless, it was observed that at least a 10-fold higher MOI of Ad/EUI-EGFP was required to achieve an equivalent response to that of the Ad/SIQ-EGFP-infected cells (Figure 5A). These findings were further confirmed through western blotting using an anti-EGFP antibody. Under equal MOI conditions (1,000 MOI), Ad/SIQ-EGFP exhibited a far more sensitive response to Teb compared with Ad/EUI-EGFP (Figure 5B).

Intravenously transferred adenovirus has been reported to be highly infectious to the liver, where the virus initially reaches via the hepatic vein and infiltrates hepatic cells.[45] Therefore, to test whether Ad/SIQ-EGFP used for HaCaT keratinocyte infection is also applicable in *in vivo* research, we injected 100 μL of adenovirus with an increased concentration, ranging from 10^6 to 10^8 virus particles per microliter into the tail vein of mouse. After 24 h of Ad/SIQ-EGFP infection, 100 μL PBS or the same amount of Teb (1 mmol/L) dissolved in PBS was infused once a day at 24-h intervals by intraperitoneal injection. One day after the chemical injection, we eviscerated the liver to examine the expression of EGFP by exploiting immunostaining with an anti-GFP antibody. In contrast with the PBS mock-injected groups, the liver exhibited positive immunostaining in response to Teb (1 mmol/L) stimuli only after infection with over 10^9 virus particles (10^7 virus particles per microliter, 100 μL injection) (Figure 5C). As expected, the Teb treatment did not elicit any signals in the liver without prior virus infection (Figure 5C). More important, virus infection at high dosages in the absence of Teb treatment did not induce any detectable background expression of EGFP (Figure 5C). To evaluate the long-term potentiation of SIQ *in vivo*, we compared EGFP transgene expression between constitutively active Ad/EGFP under the control of a CMV promoter and Teb dependent induction of Ad/SIQ-EGFP. After the delivery of an equal amount of virus particles (10^7 virus particles per microliter, 100 μL injection), Teb (1 mmol/L) was administered intraperitoneally to the Ad/SIQ-EGFP infected group twice at a 1-day interval, starting 24 h after virus infection (Figure 5D). The comparable level of the EGFP was robustly observable until 7 days after virus infection in both groups. It dramatically reduced after 14 days and became undetectable by day 21 (Figure 5D). Importantly, we did not detect any discernible liver defects in mice with virus infection using Ad/SIQ-EGFP, even after long-term regular administration of Teb (1 mmol/L) for 3 weeks (Figure S3). However, the limited sustainability of Ad/SIQ in rodent liver would hinder its long-term usage in *in vivo* experiments. Collectively, our data suggest that the SIQ system, when constructed with appropriate transgenes, can be adopted in various cultured cells and animal tissues without requiring any further modifications to toggle the transgene in a Teb-dependent manner. Furthermore, it is noteworthy that the SIQ toggle switch surpasses

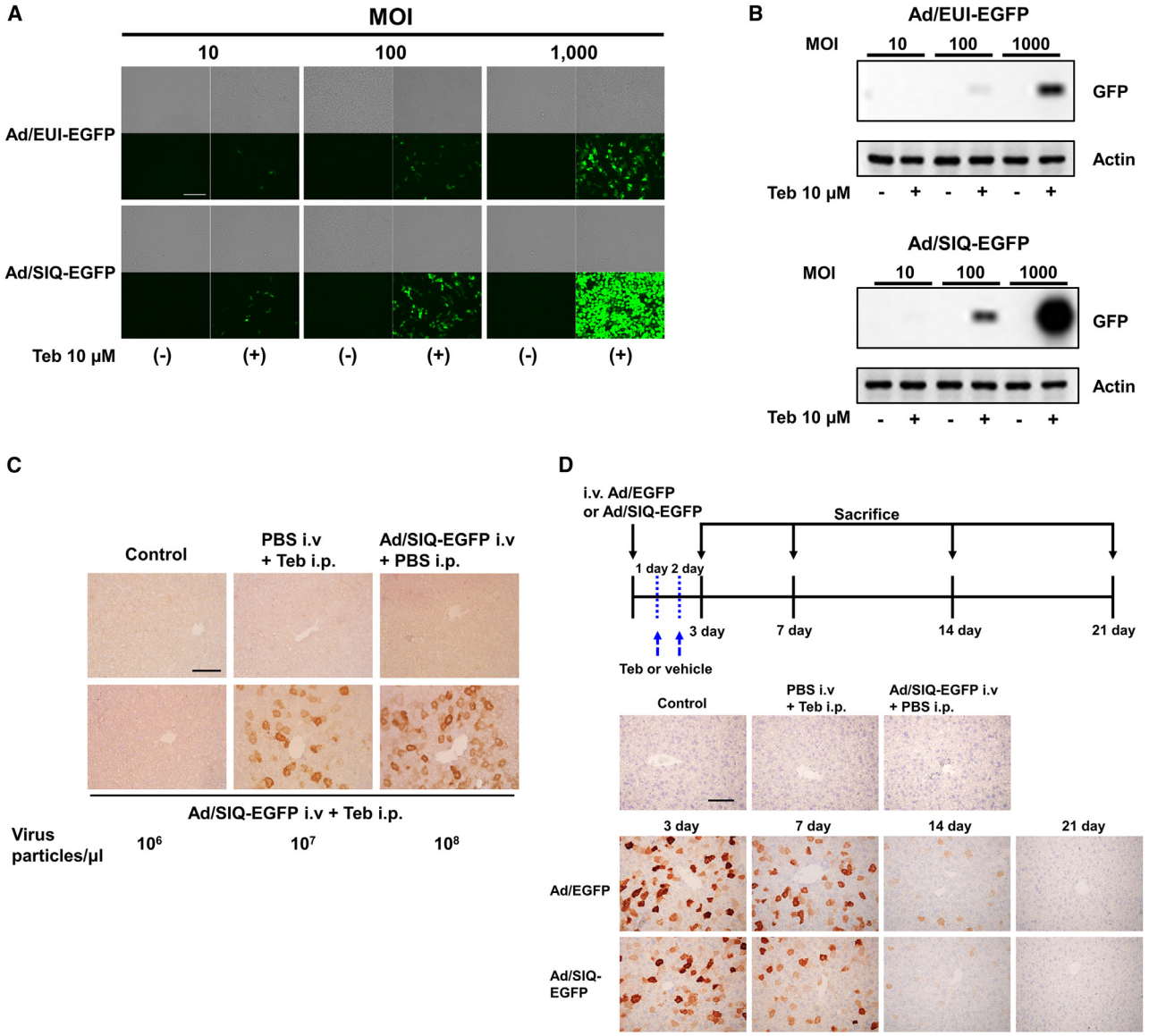


Figure 5. An adenoviral SIQ toggle switch induced strong transgene expression in HaCaT cells and mouse liver

(A) Direct comparison of adenoviral Gal4/UAS (Ad/EUI-EGFP) and SIQ (Ad/SIQ-EGFP) in HaCaT keratinocytes. Cells were infected with adenovirus at the indicated MOI and subsequently exposed to 10 μM Teb. Bright field images are shown above the GFP channel images. Scale bar, 200 μm. (B) Revalidation of fluorescence data from (A) by western blot analysis using an anti-EGFP antibody. Endogenous expression of β-actin served as the loading control. (C) *In vivo* analysis of EGFP expression in the liver after intravenous injection of increased Ad/SIQ-EGFP particles (100 μL), as indicated. Mice were treated with Teb by i.p. injection (100 μL, 1 mmol/L) once a day at 24-h intervals for 2 days. PBS alone was used as a control. EGFP expression in the liver was analyzed by immunostaining with an anti-EGFP antibody. (D) The durability of Ad/SIQ in the mouse liver. One day after injection of 100 μL Ad/SIQ-EGFP (107 virus particles/μL), Teb was administered twice (100 μL, 1 mmol/L) at a 24-h interval via i.p. injection. Mice were sacrificed at the indicated time points for the analysis of the expressivity of the EGFP transgene in the liver. Constitutively active Ad/EGFP under the control of the CMV promoter was used as a positive control. For the negative controls, indicated combinations of Ad/SIQ-EGFP and mock vehicles were delivered into the mice through the same procedures as the experimental group, but their livers were removed at day 3 only for immunostaining against EGFP. i.p., intraperitoneal injection; i.v., intravenous injection. Scale bar, 100 μm.

the Gal4/UAS-based EUI system[14] and is comparable with the CMV-derived constitutive expression system *in vivo*. This is attributed to its heightened sensitivity to chemical stimuli, lower basal transgene expressivity, and greater versatility.

Sophisticated transgene toggling can be achieved by the combination of SIQ and cumate gene switch

Although SIQ has shown its high versatility in human-derived cell lines and rodent tissues, there is room for improvement by

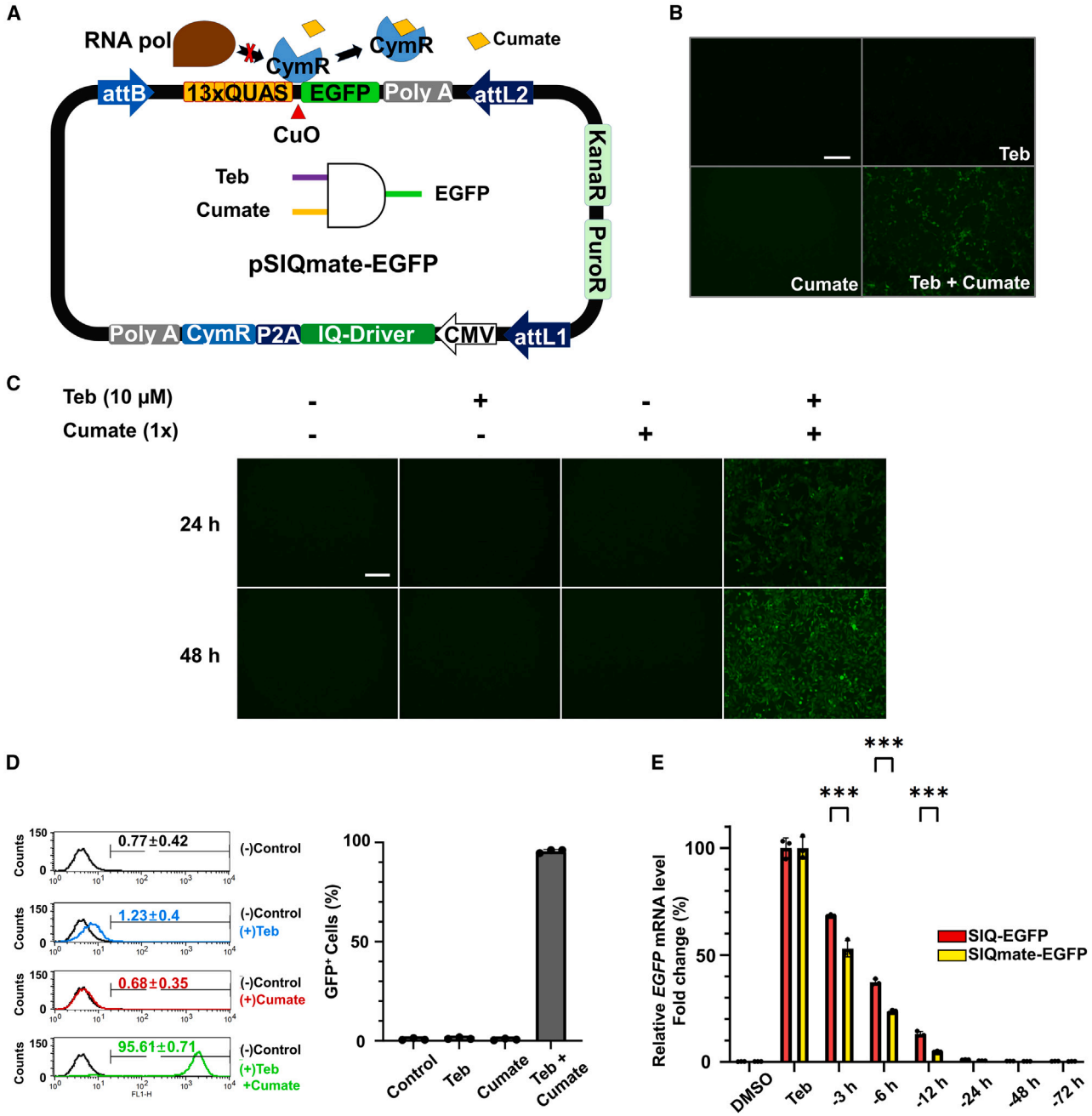


Figure 6. Accelerated reversibility of SIQmate

(A) Schematic diagram of the SIQmate system. CymR and IQ-Driver are constitutively expressed by the CMV promoter, linked by a viral P2A peptide. CuO is strategically positioned directly upstream of a transcriptional start site. CymR binds to the CuO element, suppressing transgene expression by physically hindering RNA polymerase progression. Cumate interferes with CymR binding to CuO, enabling transgene induction. Therefore, the SIQmate switch requires two orthogonal chemical inducers, Teb and cumate, for transgene activation. (B) Transient transfection of HEK293 cells with pSIQmate-EGFP. After 24 h of incubation, the transfectants were treated with the indicated chemical inducers for an additional 24 h. Fluorescence was observed under the microscope. Scale bar, 200 μm. (C) Responsiveness of an established stable cell line with SIQmate-EGFP to concomitant treatment of Teb and cumate as indicated. Prolonged incubation of the cells with both chemical stimuli increased the strength of EGFP signals. Scale bar, 200 μm. (D) FACS analysis of the established SIQmate-EGFP cell line after chemical stimulation. (Left) Segregation of cells after chemical treatment, as indicated, based on the strength of EGFP fluorescence. Quantitative analysis of the yield of EGFP-positive cells is presented in the right panel. Cells were treated with 10 μM Teb and 1× cumate for 24 h before sorting the EGFP-positive cells (n = 3). (E) Direct comparison of the rapidity of transcript disappearance between SIQ and SIQmate

(legend continued on next page)

incorporating other gene regulatory elements that can be controlled by other chemical ligands. One intriguing approach involves the development of an orthogonal gene switch, where two or more different inducers can regulate a transgene without interfering with each other. In general, chemical-based gene switches cannot be rapidly turned off due to the lingering effects of chemical inducers on the transgene in cells, even after thorough washing. As a result, the cells equipped with the chemical switches require a significant lapse of time to reinstate an inert stage.

To achieve accelerated suppression of the target gene expression, we introduced a cumate-dependent transgene repressor into the SIQ toggle switch.[46] The cumate-based gene switch consists of two major components: a CymR repressor module and CuO sequences, to which the CymR binds to suppress the transgene expression. The 4-isopropylbenzoic acid, called cumate, directly binds to CymR, detaching it from the CuO, resulting in the derepression of transgene expression.[46] We added a 28-base pair length of CuO sequences directly upstream of a putative transcription start site of the transgene of pSIQ. Additionally, we linked the SIQ driver module of pSIQ to CymR by virtue of the P2A self-cleaving 2A peptide. This arrangement allows for the simultaneous expression of both the SIQ driver and the CymR repressor. For the convenience of usage, we added Cla I, Afl II, Not I, and Sca I as MCS. We designated the vector as pSIQmate (GenBank accession number: OR355469), consisting of 9,001 nucleotides.

To test the usability of pSIQmate for transgene tuning, we inserted the EGFP reporter downstream of the CuO sequence at the MCS, resulting in the construct named pSIQmate-EGFP (Figure 6A). Following Boolean logic, the SIQmate device functions as an AND gate, requiring both Teb and cumate as separate inputs to produce an output of transgene expression. To investigate the responsiveness of SIQmate to both inducers, we transiently transfected pSIQmate-EGFP into HEK293 cells and then treated them with Teb or cumate individually, as well as in combination. The transfectants administered with Teb or cumate alone failed to elicit EGFP fluorescence. However, when both chemicals were treated concomitantly, the cells exhibited EGFP fluorescence (Figure 6B).

Subsequently, we integrated the construct into the attP site of HEK293-attP-Bla through co-transfection with phiC31 serine integrase. After validating the correct insertion of pSIQmate-EGFP using genomic PCR, we analyzed the selected clones (SIQmate-EGFP). The SIQmate-EGFP clones exhibited EGFP fluorescence only after treatment with both Teb and cumate, while either single agent alone was insufficient to elicit any detectable fluorescence signals (Figure 6C). We quantitatively evaluated the responsiveness of SIQmate-EGFP to both chemicals in combination or individually using FACS. When both Teb and cumate were administered together, more than 95% of strongly EGFP-positive cells were sorted out, while none of the other groups treated with mock or cumate alone showed any detectable fluorescence through FACS (Figure 6D). The SIQmate-EGFP clone treated with Teb alone showed a marginal elevation in fluorescence signals, which were not strong enough to be detected under fluorescence microscopy (Figures 6B and 6C). This phenomenon was quantified again through the measurement of Luciferase activity (Figure S4). This is likely due to the intermittent detachment of CymR from the CuO irrespective of cumate, which is a common occurrence among transcription regulators, as their binding to *trans*-acting elements is considered a stochastic process.[47] However, when we measured the luciferase activity after transiently transfecting both systems with *luciferase* as a transgene into the HEK293 cells, SIQmate showed significantly lower sensitivity to the chemical stimuli compared with SIQ (approximately a 0.2-fold difference) (Figure S4). Further optimization may be necessary to enhance the robustness of transgene activation in SIQmate.

These data demonstrate that the Teb-dependent IQ system and the cumate-dependent CymR system are orthogonal to each other, and thus do not influence the function of the other entity. Considering the fact that CymR acts as a latch to lock up the expression of the transgene in the absence of cumate, we assume that simply withdrawing both Teb and cumate will promptly suppress transgene activation. Compared with the delayed disappearance of *EGFP* mRNA transcribed from a SIQ-EGFP-stable cell line, the *EGFP* transcript from SIQmate-EGFP cells exhibited accelerated dampening, which is observable as early as 3 h after the removal of Teb and cumate (Figure 6E). It is noteworthy that the two discrete gene switches were inserted at an identical attP locus in the HEK293 cells. Considering the relatively long half-life of *EGFP* mRNA (~7 h),[48] the tenaciously remaining *EGFP* mRNA in SIQmate-EGFP cells might be attributed to its prolonged stability rather than *de novo* synthesis of the mRNA. Thus, we tested the tunability of SIQ and SIQmate by adopting an unstable protein, luciferase, in mammalian cells, whose half-life was reported to be at best 2 h.[49] After the transient transfection of Flag-tagged luciferase under the control of SIQ and SIQmate for 24 h, the cells were treated with Teb (10 μM) alone or with an additional 1× cumate, respectively, for another 24 h. After washing out chemical inducers, the cells were harvested at the 3, 6, and 9 h for the immunoblotting with an anti-Flag antibody (Figure S5). Although the level of transgene excitability was noticeably lower in SIQmate than in SIQ, the transgene products were completely eliminated in SIQmate as early as 3 h after withdrawal of the chemical stimuli. Therefore, suppression of lingering activity of extraneous inducers inside the cells, which has been regarded as a main conundrum of chemical-based gene switches, could be achieved by merging the CymR repressor with SIQ. Taken together, our results propose that the SIQ and

systems after removing both Teb and cumate. Established cell lines of SIQ-EGFP and SIQmate-EGFP integrated at the attP locus of HEK293 cells were treated with both chemicals for 24 h. After the removal of the chemical stimulators by thorough washing, the EGFP transcript levels were measured by RT-qPCR at the indicated time points. Notably, the reduction in EGFP mRNA was more prominent under the control of SIQmate than that of SIQ. Statistical significance was addressed using two-way ANOVA with Šidák correction (n = 3). Scale bar, 200 μm. The data are presented as mean ± SD. ***$p < 0.001$.

SIQmate gene toggling systems are attractive molecular devices highly applicable for generating a sophisticated circuitry for the fine-tuning of transgene expression.

DISCUSSION

There are several criteria that ought to be considered before designing a novel, chemically based transgene toggle switch. Any system for the regulation of transgenes should not be toxic to the cells and organisms, must be exquisitely sensitive and precisely modulated by proper drug administration or withdrawal, a smaller size of its regulatory elements is generally preferable to minimize possible immunogenic problems, and the triggering cue should be orthogonal, thereby making it possible to implant the newly developed system into other discrete toggle gene switches to create a *de novo* logic operator applicable to synthetic biology.

The fatal flaw of the binary feature of the transgene toggle switches is that it is hardly expected to achieve equal delivery of two separate vectors into the target cells or tissues simultaneously.[50] This results in an emerging population of cells with unevenly distributed plasmids, which may inevitably cause ambiguous conclusions of experiments due to the heterogeneity of the cells. Although researchers can circumvent the production of the undesirable heterogenic population of cells by establishing a stable cell line going through at least two rounds of consecutive antibiotic selection procedures, it is laborious and time consuming. Therefore, the manufacturing of a reliable and versatile singular transgene switch would be desirable in the field of preclinical as well as biological research.

Since the native QF transcriptional activator was highly toxic in fruit flies and zebrafish,[28–30,34] the QF had to be extensively amended before being adopted in animals to eliminate its cellular toxicity. We previously minimized the cytotoxicity issues of QF by removing virulent modules from the middle domain to the C-terminal end of large transactivation domain.[34] We retained only the N-terminal DBD with a small activation domain, and then added an additional identical small activation domain in tandem with a minimal VP16 activation domain and a modified EcR sequentially.[34] The finalized inducible IQ-Switch did not show any discernible embryonic toxicity in zebrafish, but retained transcriptional activity upon stimulation of Teb in a dosage-dependent manner. We achieved highly potentiated transcriptional activity of the IQ-Switch by lengthening QUAS repeats from 5 to 13.[34] After previously validating the *in vivo* value of the IQ-Switch compared with Gal4/UAS,[34] we combined the binary feature of the IQ-Switch into one (SIQ) for more convenient usage in both *in vitro* and *in vivo* studies. We demonstrated that the SIQ toggle switch is superior to the Gal4/UAS-based EUI system[14] in terms of its higher sensitivity to chemical stimuli, lower basal transgene expressivity, and greater versatility. The SIQ system finds wide applications, including transient transfection experiments, establishment of stable cell lines, and production of adenovirus, all of which require only a single round of subcloning the genes of interest into pSIQ.

The SIQ toggle switch, encompassing all the prerequisite elements in a single vector for transgene toggling, did not show any discernible leakiness of the transgene. This was experimentally validated by employing a bacterial gene, *nitroreductase*, which could convert the innocuous prodrug MTZ into a highly virulent metabolite that induces cell death (Figure 4).[51–53] Even under high concentrations of MTZ, the cells harboring SIQ remained unaffected without treatment of Teb (Figure S1B). Notwithstanding the several-fold increase in robustness of the SIQ switch compared with Gal4/UAS (Figures 2A and 2B), the leakiness of SIQ was far less than that of Gal4/UAS (Figure 2C). To expand the versatility of SIQ, we used commercially available adenovirus packaging systems (pAd/PL-DEST from Invitrogen), capable of accommodating approximately 7.5 kb of DNA. Considering the 4.5 kb of transgene regulatory modules of SIQ, including attL1 and attL2 recombination sites, we envisioned that less than 3 kb of transgene could be incorporated into the pSIQ vector for adenovirus production. The adenovirus effectively delivered the transgenes into the HaCaT keratinocyte cells, which are highly refractory to conventional transfections. The expression level of the transgenes, far better than that of Gal4/UAS, was tightly regulated by external Teb stimuli (Figures 5A and 5B). More important, the same adenovirus particle could be directly administered in rodents, where the virus-infected hepatocytes were susceptible to the intraperitoneal injection of Teb (Figure 5C). We also validated the orthogonality of SIQ in combination with another transgene inducible system responsive to a discrete small chemical inducer, cumate.[46,54,55] Both systems worked individually in cells without any detectable interference (Figures 6B and 6C). Thus, the SIQ-based singular toggle switch would become a valuable molecular and cellular tool adaptable to both preclinical investigations using adenovirus and biological research by virtue of its simplicity for transient transfection and generation of stable cell lines.

A critical concern of SIQ is the safety issues related to Teb. Although we did not observe any discernible defects in the mouse liver that had received Ad/SIQ-EGFP 2 days before the consecutive delivery of Teb for 3 weeks at a 2-day interval (Figure S3), the chemical, one of the most widely used ingredients in insecticides,[56] has not yet been approved for human use, despite being classified by the U.S. Environmental Protection Agency (https://www.federalregister.gov/documents/2016/10/14/2016-24650/tebufenozide-proposed-pesticide-tolerance) as "not likely to be carcinogenic to humans" based on the absence of evidence of carcinogenicity in animal models. Therefore, the development of druggable Teb or its agonists should precede the trial of Teb-dependent gene switches in humans. Another limitation of Ad/SIQ in *in vivo* usage is its rapid decline in rodent tissue, presumably attributed to the inherent toxicity and high immunogenicity of adenovirus.[57] Therefore, SIQ should be used in more reliable transgene delivery systems, such as AAV, to enable a more versatile application in future *in vivo* studies.

In comparison with optogenetic switches, which can swiftly toggle transgene expression by simply manipulating light illumination,[58–61] chemical-based gene switches, including SIQ, inevitably face the issue of lingering chemical inducers inside cells, even after cleaning out the

culture medium. Consequently, the remaining chemicals persistently stimulate transgene expression before the cells completely metabolize them.[62] To address the intrinsic flaws of the delayed toggling-off of SIQ after the removal of external chemical inducer Teb, we implemented CymR repressor on SIQ (SIQmate) as an inhibitory module to promptly suppress transgene expression. Cumate, also known as cumic acid, derepresses the promoter by interfering with CymR binding to the CuO sequences, which are placed immediately downstream of that promoter.[46] As shown in Figure 6E, we were able to promptly dampen the transgene expression level by simply removing Teb and cumate. In the absence of cumate, CymR binds to CuO like a latch, effectively inhibiting transcription. We did not test whether pSIQmate could be converted into an adenovirus-mediated gene delivery system due to its expanded size required to accommodate additional gene regulatory modules related to the cumate-inducible system. We proposed that SIQmate could be an alternative option for researchers when they need to hasten the shut-off of transgene expression. However, it is worth noting that the sensitivity of SIQmate to Teb is significantly weaker than that of SIQ, and is comparable with the sensitivity of Gal4/UAS.

Stable transgenic cell lines can be generated by randomly integrating a foreign gene into the genome. However, the transcriptional potency of the gene is influenced by neighboring genomic entities.[63,64] To enable a direct comparison of the cellular function of exogenous genes, it is necessary to integrate them into identical genomic loci. To achieve targeted genome insertion of the SIQ system, we used the phiC31 serine integrase after permanently landing an attP attachment site into the host cell genome. Additionally, we included a 70 bp attB site within SIQ, which the phiC31 enzyme recognizes along with attP to facilitate directional site-specific recombination.[36,65] The main shortcoming of our approach is that, for the integration of SIQ into a specific genomic locus in the cell lines of interest, researchers must pre-establish individual cell lines harboring an attP attachment site in a specific genomic region, ideally in a safe harbor locus where insertion of exogenous DNA does not interfere with genomic integrity.[66] After implanting attP into the HEK293 cells using lentivirus, we routinely integrate SIQ together with discrete transgenes into the predetermined genomic locus, and we directly validate the insertion rates using conventional genomic PCR. Serine integrase, in stark contrast with tyrosine integrase, does not require any auxiliary molecules to recombine two distinct att sites. To date, more than 4,000 predicted serine integrases have been reported.[67] Combining SIQ with other orthogonal transgene switches and serine integrases would pave the way for advanced genome engineering, gene assembly, data storage, and Boolean logic gate devices.

MATERIALS AND METHODS

Preparation of plasmids

The pSIQ plasmid was generated based on pENTR-EUI,[14] serving as the mother vector, using PCR ligation and T4 DNA polymerase (NEB) sequence and ligation-independent cloning (SLIC) methods.[68] The Gal4-VP16-EcR and 10× UAS of pENTR-EUI were substituted for the IQ-Switch driver and 13× QUAS, respectively. The pcDNA3.1 phiC31 was a gift from Dr. Konrad Basler (Addgene plasmid # 68310; http://n2t.net/addgene:68310; RRID: Addgene_68310). pSB_PhiC31LandingSite (Addgene plasmid # 48875; http://n2t.net/addgene:48875; RRID: Addgene_48875) and pEx_MCS-attBtagRFPt (Addgene plasmid # 48876; http://n2t.net/addgene:48876; RRID: Addgene_48876) were gifts from Dr. Joachim Wittbrodt. The 5×UAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y,he:tagBFP2 was a gift from Jeff Mumm (Addgene plasmid # 158651; http://n2t.net/addgene:158651; RRID: Addgene_158651). All elements required for creating the pSIQ-Switch were inserted at appropriate positions using T4 DNA polymerase SLIC. The pSIQmate plasmid was generated by directly subcloning a CymR element behind the IQ-Driver of pSIQ via a P2A peptide, and the CuO sequence was inserted at the transcription start site. The CD510B1 plasmid (pCDH-CMV-MCS-EF1-Puro) was purchased from System Biosciences. The CD510B1 lentiviral vector was modified by replacing the CMV7 promoter with an attP site using SpeI and XbaI restriction enzymes. Subsequently, the puromycin N-acetyltransferase was substituted with blasticidin S deaminase. We designated the modified vector, pBla-attP-Lenti.

Cell culture and transfection

HEK293, HEK293-derived stable cells, and HaCaT cells were cultured in DMEM supplemented with 10% fetal bovine serum (Welgene) and 1× penicillin-streptomycin (Welgene). All cells were grown at 37°C with 5% CO_2 under humidified conditions. For transfection, the Gene-Fect Transfection Reagent (Translab) was used following the manufacturer's instructions. CELENA S (Logos Biosystems) and EVOS M5000 (Invitrogen) were used for the fluorescence imaging of living cells.

Total RNA isolation and cDNA synthesis

To extract total RNA, cells were detached using a cell scraper and then centrifuged to collect them. TRI-reagent (Ambion) was added to lyse the cells, and the mixture was vigorously shaken with an additional 1/5 volume of chloroform. The agitated sample was then centrifuged, and the supernatant was transferred into a new fresh tube. Total RNA was eluted using DEPC-treated water after precipitation with isopropyl alcohol. cDNA synthesis was performed using SuperScript III (Invitrogen) following the manufacturer's instructions.

RT-qPCR

TOPreal qPCR 2× PreMIX (Enzynomics) was used to analyze the samples using Bio-Rad CFX Manager 3.1 software with the CFX Connect Real-Time PCR Detection System (Bio-Rad). The information for the primer sets is provided below.

EGFP

Forward primer: 5′-AAGCAGAAGAACGGCATCAA-3′

Reverse primer: 5′-GGGGGTGTTCTGCTGGTAGT-3′

GAPDH

Forward primer: 5′-TGCACCACCAACTGCTTAGC-3′

Reverse primer: 5′-GGCATGGACTGTGGTCATGAG-3′

Genomic DNA isolation and genomic PCR

The genomic DNA was extracted using the TIANamp Genomic DNA extraction kit (TIANGEN) following the manufacturer's instructions. Nested PCR was conducted to identify whether recombination-based stable cell lines were generated. The primer sequences are provided below.

Right arm

First PCR forward primer: 5′-AGGTCAGAAGCGGTTTTCGGG AGTAG -3′

First PCR reverse primer: 5′ – CGGACTTGAAGAAGTCGTGCTGC TTC-3′

Second PRC forward primer: 5′-TTCGGGAGTAGTGCCCCAACTG GG -3′

Second PRC reverse primer: 5′-CTTCATGTGGTCGGGGTAGCG GCTG-3′

Left arm

First PCR forward primer: 5′-GCTTCTGAGGCGGAAAGTTGAGA GGAC-3′

First PCR reverse primer: 5′-AACCCCTTGTGTCATGTCGGC GAC-3′

Second PCR forward primer: 5′-GAGAGGACATTCCAATCATAG GCTGCCC-3′

Second PCR reverse primer: 5′-CTTGTGTCATGTCGGCGACCC TACG-3′

Adenovirus preparation and transduction

Replication-incompetent adenoviruses (type 5) were generated using the Virapower adenovirus expression system (Invitrogen) following previous reports.[14] Site-specific DNA recombination between the entry vector (pENTR-EUI-EGFP or pSIQ-EGFP) and the adenoviral destination vector pAd/PL-DEST (Invitrogen) was performed using LR clonase II (Invitrogen) to generate pAd-EUI-EGFP or pAd-SIQ-EGFP. The pAd-EUI-EGFP or pAd-SIQ-EGFP plasmids were linearized by Pac 1 (New England Biolabs) digestion and then transfected into 293A cells using Lipofectamine 2000 (Life Technologies) to produce recombinant adenoviruses. Cells were grown until 80% cytopathic effect was observed, and then they were harvested to prepare recombinant adenovirus. The adenovirus particles were subsequently amplified in 293A cells, yielding crude lysates containing adenovirus particles, which were purified using the Adeno-X Maxi Purification Kit (#631533, Takara Bio). HaCaT cells were infected with Ad/EUI-EGFP or Ad/SIQ-EGFP in combination with the administration of Teb. Subsequently, the cells were analyzed by fluorescence microscopy, and cell lysates were harvested for western blot analysis.

Lentivirus preparation and infection to establish a cell line

To obtain the desired viral particles, HEK293T cells were seeded at 0.3×10^6 cells per well in six-well plates 18 h before transfection with plasmid DNA. pBla-attP-Lenti (0.45 μg) and packaging plasmids (pLP1; 0.85 μg, pLP2; 0.56 μg, pLP/VSV-G; 0.85 μg; Invitrogen) were transfected into the prepared 293T cells using Opti-MEM Reduced Serum Medium and Lipofectamine 2000 Reagent. After 48 h of transfection, the media containing viral particles were harvested and used to produce stable cells.

Stable cell line generation

To create the HEK293-attP-Bla cell line, HEK293 cells were seeded at 0.3×10^6 cells per well in six-well plates 18 h before lentiviral transduction. The HEK293 cells were then transduced with lentiviral particles using Polybrene (final concentration, 8 μg/mL). After transduction, the cells were cultured in medium containing 10 μg/mL Blasticidin for 2 weeks to select stably transduced cells. Genomic DNA was extracted from the cell lines to confirm the expression of attP-Bla by genomic PCR, with an expected product size of 466 bp.

Primers for genomic PCR

CD510B-attP-F 5′-AGGCCCGAAGGAATAGAAGA-3′. CD510B-attP-R 5′-TCTCTAGGCACCCGTTCAAT-3′. For generating pSIQ-based stable cell lines, pSIQ-EGFP, pSIQ-EGFP-P2A-NTR 2.0, or pSIQmate-EGFP plasmids were co-transfected into HEK293-attP-Bla cells along with the pCS2+/phiC31 integrase vector, respectively. After 24 h of transfection, cells were transferred to a new cell culture dish and screened with puromycin (1 μg/mL) for 14 days. Once cells formed colonies, single-cell isolation was carried out by serial dilution in 96-well plates.

Chemical treatment

Teb (Sigma-Aldrich, 31652), cumate (System Bioscience, QM100A-1), MTZ (MERCK, M3761), and z-VAD-fmk (MedChemExpress, HY-16658B) were diluted in DMEM without fetal bovine serum and penicillin-streptomycin. Subsequently, the compounds were treated to cells under various experimental conditions. In the case of cumate, the term "1× cumate" refers to a concentration of 30 μg/mL.

Western blotting

To prepare protein samples, cells were harvested and lysed using M-PER (Mammalian protein extraction reagent, ThermoFisher) supplemented with a protein inhibitor (Tech & Innovation) following the manufacturer's instructions. The protein samples were resolved under high voltage conditions using 10× G-Effect SDS-PAGE Running Buffer (Translab) and then transferred to a nitrocellulose membrane (Pall Corporation). The membrane was blocked with Phospho-block solution (Translab) for 30 min at room temperature (RT) and incubated with primary antibodies for 1 h at RT. After washing with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0), the blot was probed with a secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at RT and detected using chemiluminescence equipment (ATTO) with an ECL substrate (Translab). Antibodies for GFP (sc-8334), α-tubulin (CP06), and β-actin

(MA5-15739) were purchased from Santa Cruz Biotechnology, Calbiochem, and Invitrogen, respectively.

Fluorescence activated cell sorter

The samples were analyzed on a FACS Calibur (BD Biosciences) with Cell Quest software. Cells were washed once and then resuspended in PBS before analysis on the flow cytometer.

Caspase-3/7 assay

Cells harboring pSIQ-EGFP-P2A-NTR 2.0 were plated in 12-well plates and treated with a combination of Teb (10 μM), MTZ (10 μM), or z-VAD-fmk (20 μM) (Med Chem Express) for 24 h. Caspase-3/7 activity was measured using the Caspase-Glo 3/7 assay kit (Promega), which uses luminogenic caspase-3/7 substrates, following the manufacturer's instructions. The luminescence intensity of each sample was measured in a plate-reading luminometer (Infinite 200pro, Tecan).

Mouse care and adenoviral infection in mouse liver

Female BALB/c mice (8 weeks old) were used. In total, 100 μL vehicle (PBS) or recombinant virus solution (10^7 virus particles per microliter) was injected using a 28G hypodermic needle via intravenous injection. After 24 h of infection, 100 μL of vehicle (PBS) or the same volume of Teb dissolved in PBS (1 mmol/L) was delivered twice (once per day) into the mouse via intraperitoneal injection. Their livers were removed for immunohistochemical staining after another 24 h. For increasing dosage test, recombinant virus solution (108 virus particles/μL) was injected via intravenous injection and Teb was delivered twice (once per day). Side effects test, after virus injection, Teb was administered to mice by intraperitoneal injection (100 μL, 1 mmol/L) once a day at 48-h intervals for several days. Mice were purchased from the Biomedical Mouse Resource Center (KRIBB). All animal studies were approved (Approval No.: CNU-00114) by the Animal Experiment Ethics Committee of Chungnam National University, Korea.

Immunohistochemistry

Prepared liver samples were fixed in 10% formalin for 12 h before being embedded in paraffin for sectioning. The sectioned slides were immersed in PBS for 10 min, boiled in antigen retrieval buffer (10 mM Tris-EDTA, pH 9.0) for 20 min, and immersed again in PBS for 10 min. Finally, the slides were soaked in 3% H_2O_2 for 10 min and washed with distilled water. Then, the sections were blocked with 5% BSA in PBS for 1 h, incubated with the primary antibody against GFP (abcam183734, Abcam) for 1 h, and washed three times with 1% Triton X-100 in PBS for 3 min each time. The sections were incubated with HRP-conjugated goat anti-rabbit secondary antibody (sa002-500, Genedepot), developed using 3,3′-diaminobenzidine (brown color), and visualized with nuclear hematoxylin counterstain (blue color) under an inverted microscope (DM-IRB Leica Microsystems).

Luciferase assay

HEK293 cells prepared at 70% confluence in 12-well plates were transfected with 200 ng pEUI-Luc or pSIQ-Luc along with 20 ng pRL-CMV (Promega) using GENE-FectTM transfection reagent (TransLab Inc). After transfection with plasmid, cells were immediately treated with Teb or DMSO, and harvesting took place 8 h after the chemical treatment. The harvested transfectants were measured for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) and a Luminometer (Infinite F200, Tecan). To confirm the activity of pSIQmate-Luc, prepared HEK293 cells were transfected with 200 ng pEUI-Luc, pSIQ-Luc, and pSIQmate-Luc together with 20 ng pRL-CMV. Five hours later, cells were treated with Teb (final concentration, 10 μM) or Cumate (1×). Cells were harvested 24 h after chemical treatment, and luciferase activity was determined.

Statistical analysis

The data were represented as mean ± SD and analyzed by the Student's t test or ANOVA using Excel and GraphPad Prism software (Dotmatics). One-way ANOVA was used for comparing more than three samples, followed by Tukey's HSD test. Two-way ANOVA with Tukey's HSD or Šidák correction was exploited to analyze discrepancies between groups. A p value of less than 0.05 was considered statistically significant and stated in the figure legends. All experiments have been reproduced at least three times to ensure the reliability of the results.

DATA AND CODE AVAILABILITY

All data needed to evaluate the conclusions in the manuscript are present in the main text or the supplemental information. Plasmids will be available in Addgene (https://www.addgene.org/) or upon request. The complete sequence information of pSIQ (OR355468) and pSIQmate (OR355469) is deposited in GenBank.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101202.

ACKNOWLEDGMENTS

We thank M. Won for providing cell culture materials. This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2022R1A2C1012375, NRF-2019R1A2C1087934, NRF-2019R1A2C1084311, NRF-2022R1A2C1004076, NRF-2022R1A6A3A13072515, and NRF-2019M3F6A1109486) and by the Korean Government (MSIP) (No. 2020R1A2C2005317).

AUTHOR CONTRIBUTIONS

H.R., G.M.H., and J.R. conceived the project and wrote the manuscript. J.H. performed the experiments. K.-C.S. conducted lentivirus and adenovirus preparation and generated stable cell lines. H.-W.P. carried out *in vivo* experiments. H.J. conducted fluorescence activated cell sorter analysis. E.J. carried out caspase 3/7 assay. J.-G.L. and J.-S.L. performed molecular cloning.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

REFERENCES

1. Hu, M.C., and Davidson, N. (1987). The inducible lac operator-repressor system is functional in mammalian cells. Cell *48*, 555–566. https://doi.org/10.1016/0092-8674(87)90234-0.
2. Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA *89*, 5547–5551. https://doi.org/10.1073/pnas.89.12.5547.
3. Gossen, M., Freundlieb, S., Bender, G., Müller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science *268*, 1766–1769. https://doi.org/10.1126/science.7792603.
4. Baron, U., Gossen, M., and Bujard, H. (1997). Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. Nucleic Acids Res. *25*, 2723–2729. https://doi.org/10.1093/nar/25.14.2723.
5. Urlinger, S., Baron, U., Thellmann, M., Hasan, M.T., Bujard, H., and Hillen, W. (2000). Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc. Natl. Acad. Sci. USA *97*, 7963–7968. https://doi.org/10.1073/pnas.130192197.
6. Das, A.T., Tenenbaum, L., and Berkhout, B. (2016). Tet-On Systems For Doxycycline-inducible Gene Expression. Curr. Gene Ther. *16*, 156–167. https://doi.org/10.2174/1566523216666160524144041.
7. Kallunki, T., Barisic, M., Jäättelä, M., and Liu, B. (2019). How to Choose the Right Inducible Gene Expression System for Mammalian Studies? Cells *8*, 796. https://doi.org/10.3390/cells8080796.
8. Doshi, A., Sadeghi, F., Varadarajan, N., and Cirino, P.C. (2020). Small-molecule inducible transcriptional control in mammalian cells. Crit. Rev. Biotechnol. *40*, 1131–1150. https://doi.org/10.1080/07388551.2020.1808583.
9. Madderson, O., Teixeira, A.P., and Fussenegger, M. (2021). Emerging mammalian gene switches for controlling implantable cell therapies. Curr. Opin. Chem. Biol. *64*, 98–105. https://doi.org/10.1016/j.cbpa.2021.05.012.
10. Baron, U., and Bujard, H. (2000). Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances. Methods Enzymol. *327*, 401–421. https://doi.org/10.1016/s0076-6879(00)27292-3.
11. Osterwalder, T., Yoon, K.S., White, B.H., and Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. Proc. Natl. Acad. Sci. USA *98*, 12596–12601. https://doi.org/10.1073/pnas.221303298.
12. Esengil, H., Chang, V., Mich, J.K., and Chen, J.K. (2007). Small-molecule regulation of zebrafish gene expression. Nat. Chem. Biol. *3*, 154–155. https://doi.org/10.1038/nchembio858.
13. Kawakami, K., Asakawa, K., Hibi, M., Itoh, M., Muto, A., and Wada, H. (2016). Gal4 Driver Transgenic Zebrafish: Powerful Tools to Study Developmental Biology, Organogenesis, and Neuroscience. Adv. Genet. *95*, 65–87. https://doi.org/10.1016/bs.adgen.2016.04.002.
14. Lee, S., Sohn, K.C., Choi, D.K., Won, M., Park, K.A., Ju, S.K., Kang, K., Bae, Y.K., Hur, G.M., and Ro, H. (2016). Ecdysone Receptor-based Singular Gene Switches for Regulated Transgene Expression in Cells and Adult Rodent Tissues. Mol. Ther. Nucleic Acids *5*, e367. https://doi.org/10.1038/mtna.2016.74.
15. Vigna, E., Cavalieri, S., Ailles, L., Geuna, M., Loew, R., Bujard, H., and Naldini, L. (2002). Robust and efficient regulation of transgene expression in vivo by improved tetracycline-dependent lentiviral vectors. Mol. Ther. *5*, 252–261. https://doi.org/10.1006/mthe.2002.0542.
16. Vigna, E., Amendola, M., Benedicenti, F., Simmons, A.D., Follenzi, A., and Naldini, L. (2005). Efficient Tet-dependent expression of human factor IX in vivo by a new self-regulating lentiviral vector. Mol. Ther. *11*, 763–775. https://doi.org/10.1016/j.ymthe.2004.11.017.
17. Szulc, J., Wiznerowicz, M., Sauvain, M.O., Trono, D., and Aebischer, P. (2006). A versatile tool for conditional gene expression and knockdown. Nat. Methods *3*, 109–116. https://doi.org/10.1038/nmeth846.
18. Ferreira, M.V., Cabral, E.T., and Coroadinha, A.S. (2021). Progress and Perspectives in the Development of Lentiviral Vector Producer Cells. Biotechnol. J. *16*, e2000017. https://doi.org/10.1002/biot.202000017.
19. Nightingale, S.J., Hollis, R.P., Pepper, K.A., Petersen, D., Yu, X.J., Yang, C., Bahner, I., and Kohn, D.B. (2006). Transient gene expression by nonintegrating lentiviral vectors. Mol. Ther. *13*, 1121–1132. https://doi.org/10.1016/j.ymthe.2006.01.008.
20. Hu, P., Li, Y., Sands, M.S., McCown, T., and Kafri, T. (2015). Generation of a stable packaging cell line producing high-titer PPT-deleted integration-deficient lentiviral vectors. Mol. Ther. Methods Clin. Dev. *2*, 15025. https://doi.org/10.1038/mtm.2015.25.
21. Ortinski, P.I., O'Donovan, B., Dong, X., and Kantor, B. (2017). Integrase-Deficient Lentiviral Vector as an All-in-One Platform for Highly Efficient CRISPR/Cas9-Mediated Gene Editing. Mol. Ther. Methods Clin. Dev. *5*, 153–164. https://doi.org/10.1016/j.omtm.2017.04.002.
22. Maddalena, A., Tereshchenko, J., Bähr, M., and Kügler, S. (2013). Adeno-associated Virus-mediated, Mifepristone-regulated Transgene Expression in the Brain. Mol. Ther. Nucleic Acids *2*, e106. https://doi.org/10.1038/mtna.2013.35.
23. Cheng, S., Tereshchenko, J., Zimmer, V., Vachey, G., Pythoud, C., Rey, M., Liefhebber, J., Raina, A., Streit, F., Mazur, A., et al. (2018). Therapeutic efficacy of regulable GDNF expression for Huntington's and Parkinson's disease by a high-induction, background-free "GeneSwitch" vector. Exp. Neurol. *309*, 79–90. https://doi.org/10.1016/j.expneurol.2018.07.017.
24. Cheng, S., van Gaalen, M.M., Bähr, M., Garea-Rodriguez, E., and Kügler, S. (2021). Optimized pharmacological control over the AAV-Gene-Switch vector for regulable gene therapy. Mol. Ther. Methods Clin. Dev. *23*, 1–10. https://doi.org/10.1016/j.omtm.2021.07.007.
25. Giles, N.H., Geever, R.F., Asch, D.K., Avalos, J., and Case, M.E. (1991). The Wilhelmine E. Key 1989 invitational lecture. Organization and regulation of the qa (quinic acid) genes in Neurospora crassa and other fungi. J. Hered. *82*, 1–7. https://doi.org/10.1093/jhered/82.1.1.
26. Potter, C.J., Tasic, B., Russler, E.V., Liang, L., and Luo, L. (2010). The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. Cell *141*, 536–548. https://doi.org/10.1016/j.cell.2010.02.025.
27. Wei, X., Potter, C.J., Luo, L., and Shen, K. (2012). Controlling gene expression with the Q repressible binary expression system in Caenorhabditis elegans. Nat. Methods *9*, 391–395. https://doi.org/10.1038/nmeth.1929.
28. Subedi, A., Macurak, M., Gee, S.T., Monge, E., Goll, M.G., Potter, C.J., Parsons, M.J., and Halpern, M.E. (2014). Adoption of the Q transcriptional regulatory system for zebrafish transgenesis. Methods *66*, 433–440. https://doi.org/10.1016/j.ymeth.2013.06.012.
29. Riabinina, O., Luginbuhl, D., Marr, E., Liu, S., Wu, M.N., Luo, L., and Potter, C.J. (2015). Improved and expanded Q-system reagents for genetic manipulations. Nat. Methods *12*, 219–222. https://doi.org/10.1038/nmeth.3250.
30. Burgess, J., Burrows, J.T., Sadhak, R., Chiang, S., Weiss, A., D'Amata, C., Molinaro, A.M., Zhu, S., Long, M., Hu, C., et al. (2020). An optimized QF-binary expression system for use in zebrafish. Dev. Biol. *465*, 144–156. https://doi.org/10.1016/j.ydbio.2020.07.007.
31. Persad, R., Reuter, D.N., Dice, L.T., Nguyen, M.A., Rigoulot, S.B., Layton, J.S., Schmid, M.J., Poindexter, M.R., Occhialini, A., Stewart, C.N., Jr., and Lenaghan, S.C. (2020). The Q-System as a Synthetic Transcriptional Regulator in Plants. Front. Plant Sci. *11*, 245. https://doi.org/10.3389/fpls.2020.00245.
32. MacDonald, I.C., Seamons, T.R., Emmons, J.C., Javdan, S.B., and Deans, T.L. (2021). Enhanced regulation of prokaryotic gene expression by a eukaryotic transcriptional activator. Nat. Commun. *12*, 4109. https://doi.org/10.1038/s41467-021-24434-9.
33. Banzai, K., Shen, P., and Kamiyama, D. (2022). A Genetic Toolkit for Simultaneous Generation of LexA- and QF-Expressing Clones in Selected Cell Types in Drosophila. Neurosci. Insights *17*, 26331055211069939. https://doi.org/10.1177/26331055211069939.
34. Hong, J., Lee, J.G., Sohn, K.C., Lee, K., Lee, S., Lee, J., Hong, J., Choi, D., Hong, Y., Jin, H.S., et al. (2021). IQ-Switch is a QF-based innocuous, silencing-free, and inducible gene switch system in zebrafish. Commun. Biol. *4*, 1405. https://doi.org/10.1038/s42003-021-02923-3.
35. Lee, S., Won, M., Hwang, R.H., Hur, G.M., and Ro, H. (2018). An Ecdysone Receptor-based Singular Gene Switch for Deliberate Expression of Transgene with Robustness, Reversibility, and Negligible Leakiness. J. Vis. Exp. *135*, e57494. https://doi.org/10.3791/57494.

36. Merrick, C.A., Zhao, J., and Rosser, S.J. (2018). Serine Integrases: Advancing Synthetic Biology. ACS Synth. Biol. *7*, 299–310. https://doi.org/10.1021/acssynbio.7b00308.

37. Lo, C.A., Greben, A.W., and Chen, B.E. (2017). Generating stable cell lines with quantifiable protein production using CRISPR/Cas9-mediated knock-in. Biotechniques *62*, 165–174. https://doi.org/10.2144/000114534.

38. Curado, S., Stainier, D.Y.R., and Anderson, R.M. (2008). Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies. Nat. Protoc. *3*, 948–954. https://doi.org/10.1038/nprot.2008.58.

39. Sharrock, A.V., Mulligan, T.S., Hall, K.R., Williams, E.M., White, D.T., Zhang, L., Emmerich, K., Matthews, F., Nimmagadda, S., Washington, S., et al. (2022). NTR 2.0: a rationally engineered prodrug-converting enzyme with substantially enhanced efficacy for targeted cell ablation. Nat. Methods *19*, 205–215. https://doi.org/10.1038/s41592-021-01364-4.

40. Kim, J.H., Lee, S.R., Li, L.H., Park, H.J., Park, J.H., Lee, K.Y., Kim, M.K., Shin, B.A., and Choi, S.Y. (2011). High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. PLoS One *6*, e18556. https://doi.org/10.1371/journal.pone.0018556.

41. Chen, Y.S., Chuang, W.C., Kung, H.N., Cheng, C.Y., Huang, D.Y., Sekar, P., and Lin, W.W. (2022). Pan-Caspase Inhibitor zVAD Induces Necroptotic and Autophagic Cell Death in TLR3/4-Stimulated Macrophages. Mol. Cell. *45*, 257–272. https://doi.org/10.14348/molcells.2021.0193.

42. Bulcha, J.T., Wang, Y., Ma, H., Tai, P.W.L., and Gao, G. (2021). Viral vector platforms within the gene therapy landscape. Signal Transduct. Targeted Ther. *6*, 53. https://doi.org/10.1038/s41392-021-00487-6.

43. Lee, C.S., Bishop, E.S., Zhang, R., Yu, X., Farina, E.M., Yan, S., Zhao, C., Zheng, Z., Shu, Y., Wu, X., et al. (2017). Adenovirus-Mediated Gene Delivery: Potential Applications for Gene and Cell-Based Therapies in the New Era of Personalized Medicine. Genes Dis. *4*, 43–63. https://doi.org/10.1016/j.gendis.2017.04.001.

44. Ruozi, B., Forni, F., Battini, R., and Vandelli, M.A. (2003). Cationic liposomes for gene transfection. J. Drug Target. *11*, 407–414. https://doi.org/10.1080/10611860310001655600.

45. Duncan, S.J., Gordon, F.C., Gregory, D.W., McPhie, J.L., Postlethwaite, R., White, R., and Willcox, H.N. (1978). Infection of mouse liver by human adenovirus type 5. J. Gen. Virol. *40*, 45–61. https://doi.org/10.1099/0022-1317-40-1-45.

46. Mullick, A., Xu, Y., Warren, R., Koutroumanis, M., Guilbault, C., Broussau, S., Malenfant, F., Bourget, L., Lamoureux, L., Lo, R., et al. (2006). The cumate gene-switch: a system for regulated expression in mammalian cells. BMC Biotechnol. *6*, 43. https://doi.org/10.1186/1472-6750-6-43.

47. Raj, A., and van Oudenaarden, A. (2008). Nature, nurture, or chance: stochastic gene expression and its consequences. Cell *135*, 216–226. https://doi.org/10.1016/j.cell.2008.09.050.

48. Sacchetti, A., El Sewedy, T., Nasr, A.F., and Alberti, S. (2001). Efficient GFP mutations profoundly affect mRNA transcription and translation rates. FEBS Lett. *492*, 151–155. https://doi.org/10.1016/s0014-5793(01)02246-3.

49. Ignowski, J.M., and Schaffer, D.V. (2004). Kinetic analysis and modeling of firefly luciferase as a quantitative reporter gene in live mammalian cells. Biotechnol. Bioeng. *86*, 827–834. https://doi.org/10.1002/bit.20059.

50. Yasutsugu, S., and Youichi, S. (2011). Gene Regulatable Lentiviral Vector System. In Viral Gene Therapy, *Ch. 14*, X. Ke, ed (Rijeka: IntechOpen). https://doi.org/10.5772/18155.

51. Lindmark, D.G., and Müller, M. (1976). Antitrichomonad action, mutagenicity, and reduction of metronidazole and other nitroimidazoles. Antimicrob. Agents Chemother. *10*, 476–482. https://doi.org/10.1128/aac.10.3.476.

52. Anlezark, G.M., Melton, R.G., Sherwood, R.F., Coles, B., Friedlos, F., and Knox, R.J. (1992). The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)–I. Purification and properties of a nitroreductase enzyme from Escherichia coli–a potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). Biochem. Pharmacol. *44*, 2289–2295. https://doi.org/10.1016/0006-2952(92)90671-5.

53. Edwards, D.I. (1993). Nitroimidazole drugs–action and resistance mechanisms. II. Mechanisms of resistance. J. Antimicrob. Chemother. *31*, 201–210. https://doi.org/10.1093/jac/31.2.201.

54. Li, F.J., Xu, Z.S., Aye, H.M., Brasseur, A., Lun, Z.R., Tan, K.S.W., and He, C.Y. (2017). An efficient cumate-inducible system for procyclic and bloodstream form Trypanosoma brucei. Mol. Biochem. Parasitol. *214*, 101–104. https://doi.org/10.1016/j.molbiopara.2017.04.007.

55. Sato, S., Kikuchi, T., Nishimura, Y., Yugami, Y., Sakuraoka, M., Kita, Y., Fukuda, T., and Kobayashi, M. (2020). Generation of mouse iPS cells using an inducible expression of transgenes via the cumate gene-switch. Anal. Biochem. *599*, 113748. https://doi.org/10.1016/j.ab.2020.113748.

56. Nakagawa, Y. (2005). Nonsteroidal ecdysone agonists. Vitam. Horm. *73*, 131–173. https://doi.org/10.1016/s0083-6729(05)73005-3.

57. Kaspar, B.K. (2009). Gene Therapy: Direct Viral Delivery. Encyclopedia of Neuroscience (Elsevier), pp. 633–639. https://doi.org/10.1016/B978-008045046-9.00012–7.

58. Deisseroth, K. (2011). Nat. Methods *8*, 26–29. https://doi.org/10.1038/nmeth.f.324.

59. Yamada, M., Suzuki, Y., Nagasaki, S.C., Okuno, H., and Imayoshi, I. (2018). Light Control of the Tet Gene Expression System in Mammalian Cells. Cell Rep. *25*, 487–500.e6. https://doi.org/10.1016/j.celrep.2018.09.026.

60. Emiliani, V., Entcheva, E., Hedrich, R., Hegemann, P., Konrad, K.R., Lüscher, C., Mahn, M., Pan, Z.-H., Sims, R.R., Vierock, J., and Yizhar, O. (2022). Optogenetics for light control of biological systems. Nat. Rev. Methods Primers *2*, 55. https://doi.org/10.1038/s43586-022-00136-4.

61. Zhou, Y., Kong, D., Wang, X., Yu, G., Wu, X., Guan, N., Weber, W., and Ye, H. (2022). A small and highly sensitive red/far-red optogenetic switch for applications in mammals. Nat. Biotechnol. *40*, 262–272. https://doi.org/10.1038/s41587-021-01036-w.

62. Politi, N., Pasotti, L., Zucca, S., Casanova, M., Micoli, G., Cusella De Angelis, M.G., and Magni, P. (2014). Half-life measurements of chemical inducers for recombinant gene expression. J. Biol. Eng. *8*, 5. https://doi.org/10.1186/1754-1611-8-5.

63. Mihelčić, M., Šmuc, T., and Supek, F. (2019). Patterns of diverse gene functions in genomic neighborhoods predict gene function and phenotype. Sci. Rep. *9*, 19537. https://doi.org/10.1038/s41598-019-55984-0.

64. Long, L., Li, X., Wei, H., and Li, W. (2020). Features of the Influence of a DNA Sequence on Its Adjacent Sequence. ACS Omega *5*, 23631–23644. https://doi.org/10.1021/acsomega.0c02264.

65. Rutherford, K., Yuan, P., Perry, K., Sharp, R., and Van Duyne, G.D. (2013). Attachment site recognition and regulation of directionality by the serine integrases. Nucleic Acids Res. *41*, 8341–8356. https://doi.org/10.1093/nar/gkt580.

66. Sadelain, M., Papapetrou, E.P., and Bushman, F.D. (2011). Safe harbours for the integration of new DNA in the human genome. Nat. Rev. Cancer *12*, 51–58. https://doi.org/10.1038/nrc3179.

67. Yang, L., Nielsen, A.A.K., Fernandez-Rodriguez, J., McClune, C.J., Laub, M.T., Lu, T.K., and Voigt, C.A. (2014). Permanent genetic memory with >1-byte capacity. Nat. Methods *11*, 1261–1266. https://doi.org/10.1038/nmeth.3147.

68. Jeong, J.Y., Yim, H.S., Ryu, J.Y., Lee, H.S., Lee, J.H., Seen, D.S., and Kang, S.G. (2012). One-step sequence- and ligation-independent cloning as a rapid and versatile cloning method for functional genomics studies. Appl. Environ. Microbiol. *78*, 5440–5443. https://doi.org/10.1128/aem.00844-12.