

# A Tunable Genetic Switch Based on RNAi and Repressor Proteins for Regulating Gene Expression in Mammalian Cells

Tara L. Deans,<sup>1</sup> Charles R. Cantor,<sup>1</sup> and James J. Collins<sup>1,\*</sup>

<sup>1</sup>Department of Biomedical Engineering, Center for BioDynamics and Center for Advanced Biotechnology, Boston University, Boston, MA 02215, USA

\*Correspondence: jcollins@bu.edu

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## SUMMARY

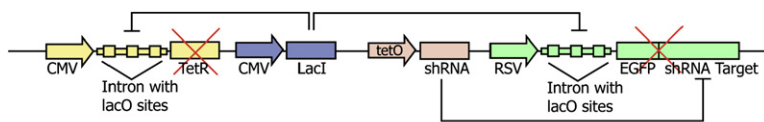
Here, we introduce an engineered, tunable genetic switch that couples repressor proteins and an RNAi target design to effectively turn any gene off. We used the switch to regulate the expression of EGFP in mouse and human cells and found that it offers >99% repression as well as the ability to tune gene expression. To demonstrate the system's modularity and level of gene silencing, we used the switch to tightly regulate the expression of diphtheria toxin and Cre recombinase, respectively. We also used the switch to tune the expression of a proapoptotic gene and show that a threshold expression level is required to induce apoptosis. This work establishes a system for tight, tunable control of mammalian gene expression that can be used to explore the functional role of various genes as well as to determine whether a phenotype is the result of a threshold response to changes in gene expression.

## INTRODUCTION

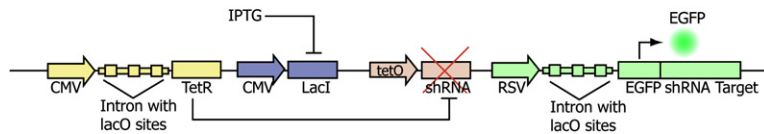
Synthetic biology is an emerging field that aspires to design and build functioning biological circuits, including gene expression systems, using well-characterized biomolecular components and genetic modules (Atkinson et al., 2003; Basu et al., 2004; Becskei and Serrano, 2000; Blake et al., 2003; Elowitz and Leibler, 2000; Elowitz et al., 2002; Fung et al., 2005; Gardner et al., 2000; Guet et al., 2002; Guido et al., 2006; Hooshangi et al., 2005; Isaacs et al., 2003, 2004; Isalan et al., 2005; Kobayashi et al., 2004; Kramer et al., 2004, 2005; Kramer and Fussenegger, 2005; Malphettes and Fussenegger, 2006; Ozbudak et al., 2002; Pedraza and van Oudenaarden, 2005; Rosenfeld et al., 2002, 2005; You et al., 2004). Generating high-fidelity and inducible gene expression systems that can operate in an intact organism would assist experimental studies of cellular function, development, and disease.

Several techniques exist to regulate gene expression; however, each carries its own caveat in function. It was discovered that both the *tetR* and *lacI* *Escherichia coli* repressor systems function in mammalian cell tissue culture and in mice (Brown et al., 1987; Gossen and Bujard, 1992; Hu and Davidson, 1987; Scrable, 2002), which proved to be a great advance in the understanding of cellular function. Other similar techniques have been used, including the inducible Gal4/UAS system, to control gene expression at the transcriptional level (Ornitz et al., 1991). While these techniques offer good repression, they exhibit leakiness that precludes the gene of interest from being completely turned off. Studies performed with double-stranded RNA in the nematode *Caenorhabditis elegans* revealed a sequence-specific RNA-mediated pathway for turning off gene expression (Fire et al., 1998; Guo and Kemphues, 1995). This process, known as RNA interference (RNAi), has been adapted for use in tissue culture and mammals with the introduction of small interfering RNAs (siRNAs) and short-hairpin RNAs (shRNAs). RNAi has revolutionized biological research; however, targeting locations on mRNAs for robust knock-down is empirical and often requires screening very large numbers of selected mRNA sequences (Paddison et al., 2004). Additionally, off-target effects can affect genes not related to the gene of interest. A commonly used method to activate or inactivate gene expression in mice involves the use of site-specific *cre* recombinase (*cre*). *cre*, which was derived from bacteriophage P1, mediates the deletion of a DNA sequence flanked by a pair of *cre* recognition sequences, called *loxP* sites (Sternberg and Hamilton, 1981). A disadvantage of this approach is that it is dependent on the coexpression of the transgene *cre*, which causes a permanent genetic event, restricting any regulation of gene expression. This approach can be made inducible with the application of *cre*-ERTM or ERT2 fusion proteins; however, the inducing ligand, tamoxifen, can be toxic at the dosage levels required for recombination (Danielian et al., 1998; Imai et al., 2001). The caveats associated with these systems for regulating gene expression make it difficult to study many fundamental questions concerning cellular processes and diseases.

## A. Switch Off (No Induction)



## B. Switch On (Induced)



(B) In the induced state (LTRi-EGFP in the on state), isopropyl- $\beta$ -thiogalactopyranoside (IPTG) binds to the LacI proteins, producing a conformational change in the repressor proteins. This causes them to no longer bind to the *lac* operator sites, which allows for the transcription of *EGFP* and *tetR*. The Tet repressor proteins bind to the *tet* operator site located in the U6 promoter of the RNAi module, repressing the transcription of the shRNA. The resulting effect is robust expression of *EGFP*.

To address this problem, we have developed within the context of synthetic biology a tunable mammalian genetic switch. We engineered the switch by creating a synthetic gene network that couples repressor proteins with an RNAi design involving shRNA; we call this switch LTRi, which stands for *Lac-Tet-RNAi*. Gene expression is turned on by adding an inducer, isopropyl- $\beta$ -thiogalactopyranoside (IPTG), which controls the repressor elements at the transcriptional level, while simultaneously turning off the RNAi component to allow the transcript to be retained and translated. The switch is modular in nature, allowing for the regulation of any gene.

We used the genetic switch to regulate *EGFP* (LTRi-*EGFP*) in chinese hamster ovary (CHO) cells and human embryonic kidney 293 (HEK293) cells and found that it offers >99% repression compared to positive controls. In addition to sustaining a tight off state, LTRi also exhibits tunable, reversible control of gene expression. We used LTRi to control the expression of the highly toxic  $\alpha$  chain of diphtheria toxin, *DTA* (LTRi-*DTA*), in order to demonstrate the system's modularity and level of gene silencing. We found that cells with LTRi-*DTA* in the off state survive despite the presence of the *DTA* gene and can be triggered to die following induction with IPTG. To demonstrate the potential for other *in vivo* experimental applications, we used the switch to control the expression of Cre recombinase (LTRi-*cre*) in *Rosa26* primary mouse cells. Similar to the LTRi-*DTA* results, we found that cells with LTRi-*cre* in the off state exhibit no expression of the *cre* transgene, and genetic recombination is observed only following induction of the switch with IPTG. To explore whether LTRi is capable of regulating a biological process, we put *bax*, a proapoptotic gene, under its control (LTRi-*bax*). *Bax* is a member of the Bcl-2 family of proteins that has been associated with apoptotic death both in cell culture and in intact animals (Aneja et al., 2006; Pastorino et al., 1998; Shinoura et al., 1999). The overexpression of *Bax* has been shown to cause apoptosis by

### Figure 1. Schematic Diagrams of the Synthetic Gene Network

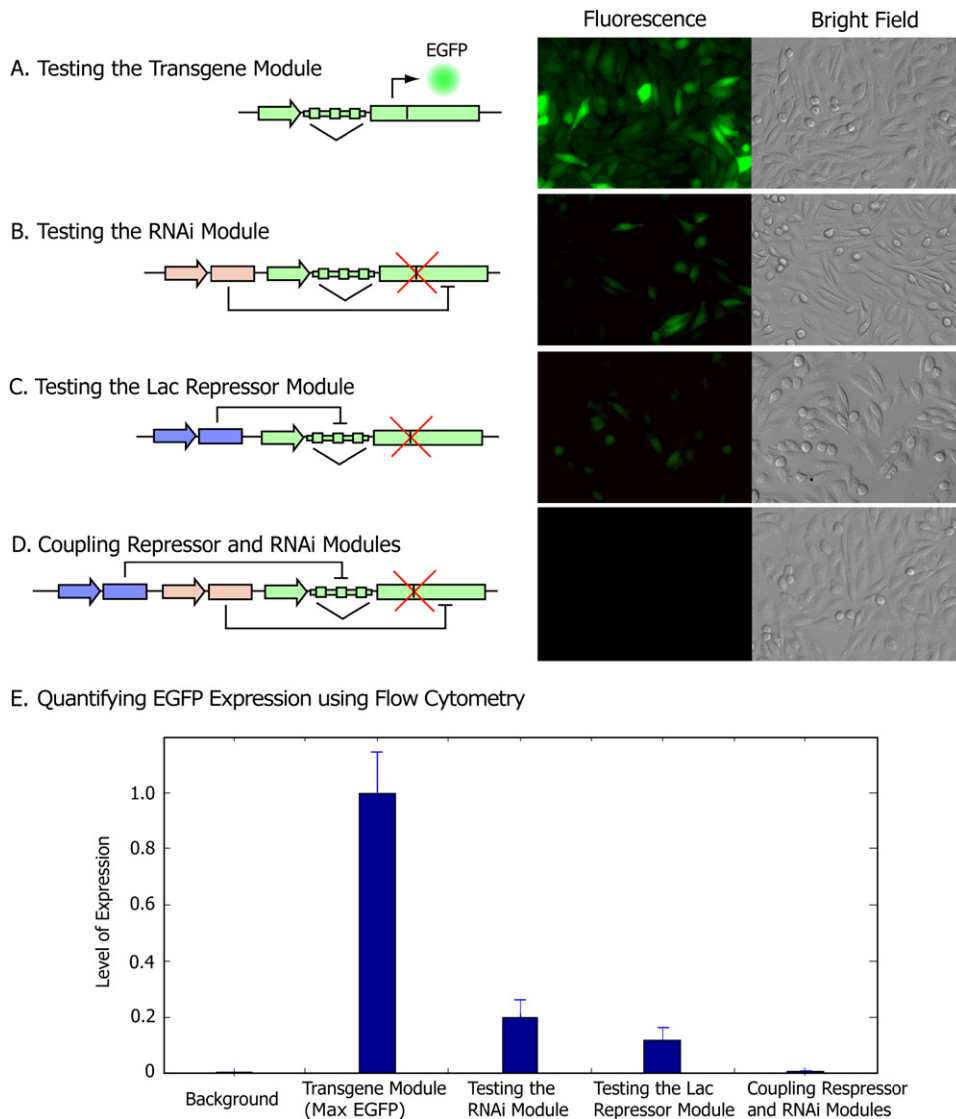
(A) In the uninduced state (LTRi-*EGFP* in the off state), LacI repressor proteins are constitutively expressed (blue) and bind to the *lac* operator sites in the transgene module (green). This causes transcriptional repression of *EGFP*. The LacI repressor proteins also bind to the *lac* operator sites in the *tetR* repressor module (yellow), which causes transcriptional repression of *TetR*. With the repression of *TetR*, shRNA is transcribed by the U6 promoter and complementarily binds to the synthetic target sequence located in the 3'UTR of the *EGFP* mRNA. Any *EGFP* leakage of the transcript is targeted by the RNAi module (pink), resulting in undetectable *EGFP* expression.

disrupting the mitochondrial transmembrane potential and releasing cytochrome *c* from the mitochondria (Katiyar et al., 2005; van Engeland et al., 1998). By tuning the level of *Bax* in primary mouse cells, we show that a threshold level of *Bax* is required to induce apoptosis. This work establishes a system for tight, tunable, reversible control of mammalian gene expression that can be used to explore the functional role of developmental genes and mechanisms of biological switches, as well as to assay threshold responses to changes in gene expression.

## RESULTS

### Design of the Genetic Switch

We initially used our genetic switch to regulate the expression of enhanced green fluorescent protein, *EGFP* (LTRi-*EGFP*). LTRi-*EGFP* was designed so that in the off state (Figure 1A), LacI provides transcriptional repression of *EGFP* and any leakage of the *EGFP* transcript is knocked down by the RNAi component of the switch. Specifically, the *lacI* repressor module produces LacI proteins that bind to the *lac* operator sites located in an intron adjacent to *EGFP* in the transgene module. Any *EGFP* mRNA that is transcribed as a result of incomplete repression is subsequently targeted for degradation by shRNA produced by the RNAi module. The shRNA molecules target the mRNA of *EGFP* via a synthetic target sequence that was placed in the 3'UTR. In addition to repressing the transcription of the transgene, the LacI proteins also repress the second repressor module in the system, the *tetR* repressor module. This component controls the functionality of the RNAi module by regulating the transcription of shRNA molecules that target the mRNA of *EGFP*. The genetic switch can be flipped to the on state (Figure 1B) by adding IPTG, which binds to LacI and produces a conformational change in the repressor proteins, causing them to no longer bind to the *lac* operator sites. This allows



**Figure 2. Building and Testing the Genetic Switch in a Modular Fashion**

In (A)–(D), the first column includes a schematic diagram of the tested module, while the second and third columns include fluorescence and bright-field microscope images, respectively, of cells containing the tested module.

(A) The transgene module served as our positive control (corresponding to maximum EGFP expression).

(B) The RNAi module was added to the transgene module to characterize its functionality. Microscope images show that RNAi alone is incapable of suppressing EGFP expression to undetectable levels.

(C) The *lacI* repressor module was added to the transgene module to characterize its functionality. Microscope images show that transcriptional repression alone is incapable of suppressing EGFP expression to undetectable levels.

(D) The *lacI* repressor and RNAi modules were added together to the transgene module to characterize their combined functionality. Microscope images show that there is effectively no EGFP expression when the transcriptional and posttranscriptional mechanisms are coupled together.

(E) To quantify the expression of EGFP, we repeated the experiments described in (A)–(D) using flow cytometry. All EGFP expression levels were normalized to the maximum EGFP expression level. Each bar represents the mean EGFP expression in eight independent experiments. In this and all other figures, error bars represent standard deviations.

for the transcription of *EGFP* and *tetR*. The Tet repressor proteins bind to the *tet* operator site located in the U6 promoter of the RNAi module, which represses the transcription of shRNA. The resulting effect is robust expression of EGFP.

### Construction and Functional Characterization of the Genetic Switch

We constructed LTRi-EGFP in a modular fashion and tested it using microscopy and flow cytometry (Figure 2). First, we designed a posttranscriptional module

that uses a U6 promoter containing a *tet* operator site to transcribe a 42 nucleotide RNA transcript. This transcript forms a shRNA and has complementarity to a 19 nucleotide sequence in the *E. coli*  $\beta$ -galactosidase sequence. This 19 nucleotide target sequence was placed in the 3'UTR of the *EGFP* gene to be targeted by the shRNA, giving rise to a system that is not gene specific, allowing for any transgene to be controlled by the module. Furthermore, by designing the target sequence to have complementarity to  $\beta$ -galactosidase, we minimized any potential off-target effects that could be caused by the RNAi module. Using transient transfections, we compared the expression of EGFP with and without the target and found that the presence of the target sequence in the 3'UTR did not significantly change the expression of EGFP. As a result, we used the transgene module controlling the expression of EGFP with the target sequence (Figure 2A) as our positive control (corresponding to maximum EGFP expression).

Next, we placed the RNAi module on the same plasmid as the transgene module to test the effectiveness of RNAi alone (Figure 2B). Using transient transfections, we found that this system offers approximately 80% knockdown of the transgene (Figure 2E) compared to the positive control. Microscope images confirmed that RNAi alone is incapable of completely shutting off EGFP expression (Figure 2B). We tested other target sequences (e.g., to luciferase) and obtained similar results. To ensure that the shRNA effect was due to targeting the 3'UTR sequence, we placed the RNAi module onto a plasmid that expressed EGFP but lacked the target sequence in the 3'UTR. Importantly, we observed no change in the expression of EGFP, indicating that the shRNA did not exhibit off-target effects with respect to EGFP.

We then placed the *lacI* repressor module on the same plasmid as the transgene module to examine the effectiveness of repression alone (Figure 2C). Using transient transfections, we found that this system offers approximately 85% repression (Figure 2E) compared to the positive control. Similar to the RNAi module, microscope images showed that transcriptional repression alone is incapable of completely shutting off EGFP expression (Figure 2C).

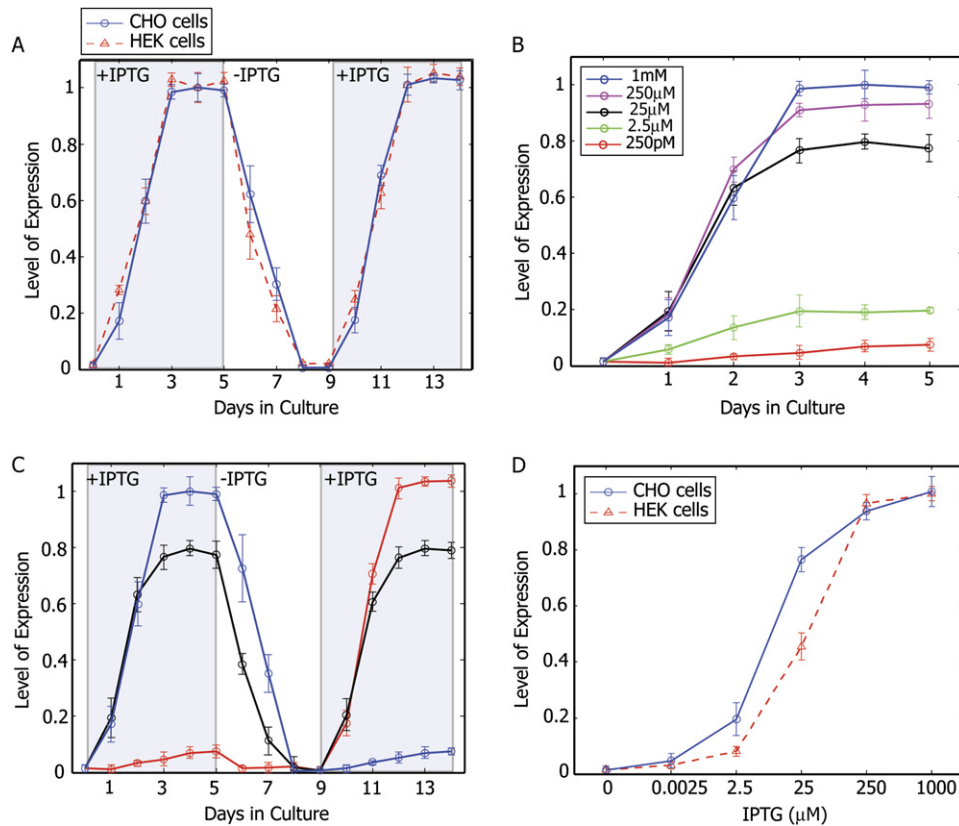
Finally, we coupled the transcriptional and posttranscriptional mechanisms by placing both the *lacI* repressor module and the RNAi module on the same plasmid as the transgene module (Figure 2D). We found that this system offers greater than 99% repression (Figure 2E) compared to the positive control. Microscope images confirmed that there is effectively no EGFP expression when the transcriptional and posttranscriptional mechanisms are coupled together (Figure 2D). To complete our synthetic gene network, we added the last module, the *tetR* repressor module (Figure 1). As noted above, this module acts to repress the transcription of the shRNA molecules from the RNAi module.

### Characterizing the Dynamics and Tunability of the Genetic Switch

Next, we stably transfected LTRi-*EGFP* into CHO cells to characterize the dynamics of the system. Using flow cytometry, we studied the switching-time characteristics of the network over a 14 day period (Figure 3A, solid blue line). After 3 days of induction, the system was fully induced, exhibiting expression levels approximately 85-fold greater than basal levels. When the inducer was removed, full repression/knockdown was observed in 3 days. The system could be repeatedly and reversibly induced on and off with the addition and removal of IPTG (Figure 3A). To validate the levels of EGFP protein expression, immunoblots were performed on the specified days of induction (see Figure S1A in the Supplemental Data available with this article online). Consistent with our flow cytometry results, the immunoblots showed no detectable EGFP protein in the absence of inducer and a gradual increase in EGFP protein up until day 3 of induction. Our flow cytometry data (Figure S1B) also showed that the induction of the switch was uniform across the cells, i.e., an induction level of 50% arose from all cells expressing at approximately 50% of the maximum.

We also added different amounts of IPTG and showed that our switch can be used to tune the level of EGFP expression (Figure 3B). We found that EGFP expression can be maintained at different levels by varying inducer concentrations. To further examine the flexibility and control maintained by LTRi, we chose three induction levels: high (1 mM IPTG), medium (25  $\mu$ M IPTG), and low (250 pM IPTG). After full induction at each of these levels, the expression of EGFP was turned off (Figure 3C). The cells originally at the high induction level (1 mM IPTG) were reinduced with 250 pM IPTG, and the cells originally at the low induction level (250 pM IPTG) were reinduced with 1 mM IPTG. The cells originally at the medium induction level (25  $\mu$ M IPTG) were reinduced at the same level. We found that we could predictably manipulate the expression of EGFP by changing the amount of inducer, independent of the expression history of the switch (Figure 3C). Together, these results show that LTRi can precisely control the expression level of individual genes in a tunable and reversible fashion.

To compare the dynamics of our switch in other cell lines, we stably transfected LTRi-*EGFP* into HEK293 cells. Our results indicate that the dynamics of LTRi is independent of cell type (Figure 3A). Moreover, we investigated dose-response levels of LTRi-*EGFP* in both CHO and HEK cells by examining different induction levels at day 3 of induction and obtained similar dose-response characteristics (Figure 3D). Likewise, we found that LTRi can be used to tune the expression of EGFP in HEK293 cells with results similar to those obtained in CHO cells (Figure S2). Together, these results show that our system can precisely control the expression level of an individual gene in a tunable and reversible fashion, regardless of cell type.



**Figure 3. The Genetic Switch Controls Gene Expression in a Tunable and Reversible Fashion**

(A) CHO cells (solid blue line with circles) and HEK293 cells (dotted red line with triangles) were stably transfected with LTRi-EGFP. Using flow cytometry, we studied the switching time over a 14 day period. The system was repeatedly induced on and off. Each data point represents the average EGFP expression in at least five independent experiments.

(B) The tunability of LTRi-EGFP stably transfected in CHO cells was studied by adding five different amounts of inducer. Each data point represents the average EGFP expression in at least five independent experiments.

(C) To examine the flexibility of the control maintained by the switch stably transfected in CHO cells, three different induction levels were chosen: high (1 mM IPTG), medium (25 μM IPTG), and low (250 pM IPTG). After full induction at each of these levels, the expression of EGFP was turned off. The cells originally at the high induction level (blue data points) were reinduced at the low level, and the cells originally at the low induction level (red data points) were reinduced at the high level. The cells originally at the medium induction level (black data points) were reinduced at the same level. Each data point represents the average EGFP expression in at least five independent experiments.

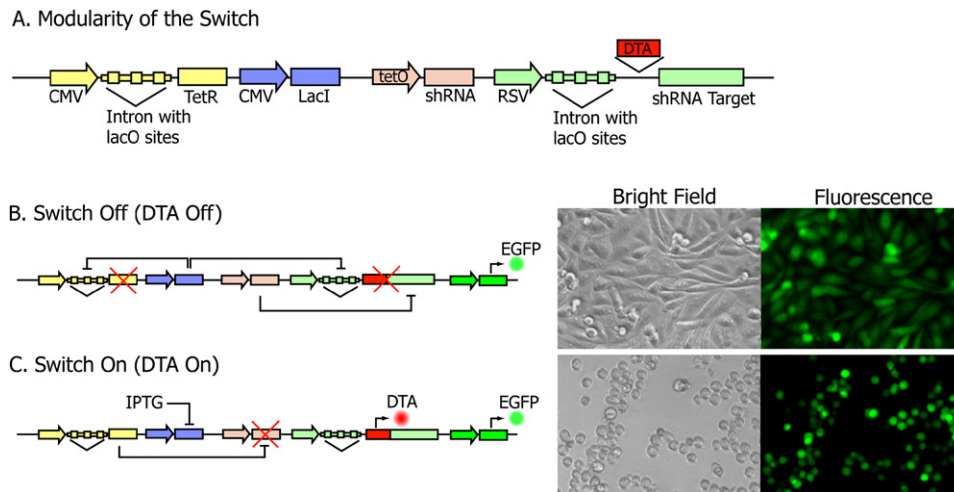
(D) An induction curve is shown for both stably transfected CHO cells (solid blue line with circles) and HEK293 cells (dotted red line with triangles). Each data point represents the mean EGFP expression in ten independent experiments.

### Demonstrating the Tight Repression and Biological Utility of the Genetic Switch

Because the regulatory elements of LTRi that are responsible for the tight control of gene expression are not associated with either a specific transgene or specific promoters, the switch is capable of controlling any gene of interest. In addition, tissue-specific promoters can be used for in vivo experiments. To emphasize this modularity and to highlight the level of gene silencing achieved by the switch, we replaced EGFP with DTA (LTRi-DTA) (Figure 4A). DTA functions by inhibiting protein synthesis (Greenfield et al., 1983), and it has been shown to be highly toxic in cells, with a single molecule being sufficient to kill a cell (Yamaizumi et al., 1978). Despite DTA's toxicity, we were able to establish stable cell lines containing the

switch controlling the expression of DTA and to grow them for more than 4 weeks. To confirm that all cells in culture contained LTRi-DTA, we added EGFP driven by a constitutive promoter to the same plasmid as the genetic switch (Figure 4A). Thus, any cell expressing EGFP also carried the switch regulating the expression of DTA. Impressively, we found that cells with the genetic switch in the off state survived despite the presence of the DTA gene (Figure 4B) and could be triggered to die following induction with IPTG (Figure 4C). Microscope images confirmed these findings (Figures 4B and 4C), which clearly demonstrated that our engineered switch can be used to effectively turn any gene off.

To explore other potential in vivo experimental applications, we used LTRi to regulate the expression of Cre



**Figure 4. The Genetic Switch Tightly Controls DTA Expression**

(A) Schematic diagram of LTRi-*DTA*.

(B) The first column shows a schematic diagram of LTRi-*DTA* in the off state, along with an *EGFP* reporter module. The second and third columns show bright-field and fluorescence microscope images, respectively, of cells with LTRi-*DTA* in the off state.

(C) The first column shows a schematic diagram of LTRi-*DTA* in the on state, along with an *EGFP* reporter module. The second and third columns show bright-field and fluorescence microscope images, respectively, of cells with LTRi-*DTA* in the on state.

recombinase (Figure 5A). LTRi-*cre* was transiently transfected into primary mouse fibroblasts derived from Rosa26  $\beta$ -galactosidase reporter cells (Soriano, 1999). In these cells, Cre recombinase activity results in the deletion of transcriptional stop elements and facilitates the expression of  $\beta$ -galactosidase (Figure S3). In this context, genetic recombination occurs, causing a permanent genetic modification, so even low levels of Cre expression can be detected histologically. In this experiment, the synthetic target in the transgene module was changed to a 19 nucleotide segment of luciferase. Cells with LTRi-*cre* in the off state showed no indication of  $\beta$ -galactosidase activity (Figure 5B). However, upon induction of the switch with IPTG, cells that were transfected with LTRi-*cre* expressed the  $\beta$ -galactosidase protein and turned blue (Figure 5C).

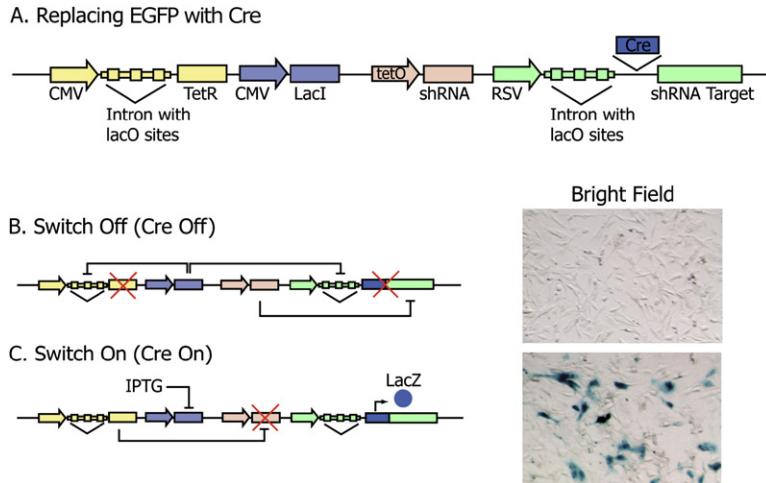
To investigate whether LTRi can also be used to regulate a biological process, we put *bax*, a proapoptotic gene, under the control of the switch (LTRi-*bax*) (Figure 6A). As previously reported, the overexpression of Bax causes cells to undergo apoptosis, and high levels of Bax protein are widely regarded as a hallmark of cells undergoing apoptosis (Pastorino et al., 1998; Shinoura et al., 1999; Wolter et al., 1997). pUC19 was transfected into Rosa26 cells to establish a basal level of apoptosis and cell death that results from transfecting the cells. All data were normalized to the level of dye retention obtained from these transfections. Transient transfections of LTRi-*bax* were performed and analyzed 48 hr after transfection at four different induction levels: 0, 250 pM, 2.5  $\mu$ M, and 1 mM IPTG. We found that at low and moderate levels of Bax expression, cells did not display increased levels of

apoptosis (Figure 6B); however, at high levels of Bax expression, there was a significant increase in apoptosis and cell death (Figure 6B). These findings indicate a possible threshold response for Bax-induced apoptosis. To ensure that cell death and apoptosis were not a result of the presence of IPTG, we exposed nontransfected cells to the indicated levels of IPTG and found no change in apoptosis or cell death (Figure 6B).

## DISCUSSION

Despite sophisticated advances in developing tools for controlling gene expression, the ability to effectively regulate a transgene of interest is still imperfect due to leakiness, poor induction levels, and irreversibility of the genetic modification. In this study, we show that these limitations can be overcome by engineering a genetic network of interacting modules for regulating mammalian gene expression. We use well-characterized, individually engineered modules to create a genetic circuit that exhibits undetectable levels of gene expression in the off state. Gene expression is triggered by the addition of IPTG, and the level of transgene expression can be precisely and reversibly tuned by adding various amounts of IPTG. Impressively, as seen with the stable cell lines containing the switch controlling *EGFP* and *DTA*, a regulated state can be transmitted as cells grow and divide.

Recently, Szulc et al. (2006) introduced a method for controlling both Pol II- and Pol III-type promoters using the KRAB domain tethered to the *tetR* repressor. This system was used to regulate the production of a transgene and shRNA to a separate target gene in the cellular



**Figure 5. The Genetic Switch Tightly Controls Cre Expression**

(A) Schematic diagram of LTRi-cre. Rosa26 cells were transiently transfected with LTRi-cre.

(B) The first column shows a schematic diagram of LTRi-cre in the off state. The second column shows a bright-field microscope image of cells with LTRi-cre in the off state.

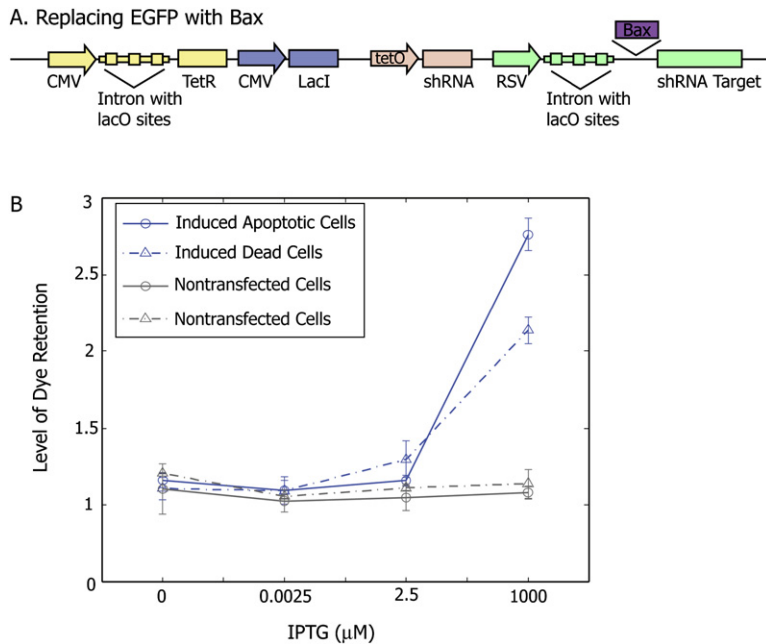
(C) The first column shows a schematic diagram of LTRi-cre in the on state. The second column shows a bright-field microscope image of cells with LTRi-cre in the on state.

genome. While this method offers an approach for regulating endogenous genes, it lacks specificity and robustness because the shRNAs target mRNA sequences of the endogenous gene of interest; this could lead, in many cases, to undesirable off-target effects.

More recently, Banaszynski et al. (2006) generated a technique for regulating protein stability in mammalian cells by fusing a 107 amino acid residue from the rapamycin-binding protein (FKBP) to a protein of interest. They showed that the residue can function as a destabilizing domain (DD) to render the protein fused to this domain unstable and thus no longer functional. The addition of a ligand protects the protein fused to the DD from degradation; therefore, the protein becomes functional with the addition of the ligand (Banaszynski et al., 2006). This system offers an approach for regulating gene expression

through a posttranslational mechanism. However, the requirement for fusion proteins is potentially an important limitation for studying native protein function. Fusion proteins often do not function like native proteins since the introduction of the additional amino acids disrupts folding, which can sterically inhibit catalytic or protein binding domains.

It is well-established that dynamic changes of gene expression levels occur throughout the life of organisms, particularly during development and the onset of disease or pathology. The tunability of LTRI makes it an ideal tool for studying the mechanisms involved in cellular processes and diseases. Currently, many genetic phenotypes are mimicked in mouse models by generating knockout animals. While this process can offer many insights based on the phenotype of an animal missing



**Figure 6. The Genetic Switch Tunes the Expression Level of Bax, Revealing a Possible Threshold Effect for Inducing Apoptosis**

(A) Schematic diagram of LTRi-bax. Rosa26 cells were transiently transfected with LTRi-bax.

(B) Quantitative flow cytometry analysis of apoptotic and dead cells using annexin V and propidium iodide (PI) staining. After 48 hr of induction at the indicated IPTG levels, cells were stained and analyzed using Alexa Fluor 488-conjugated annexin V along with PI. The apoptotic cells were Alexa Fluor 488 positive and PI negative, whereas the dead cells were PI positive. Low and moderate levels of Bax show no increase in either apoptosis or cell death, whereas high induction levels of Bax indicate a significant increase in apoptosis and cell death. To ensure that cell death and apoptosis did not result from the presence of IPTG, we exposed nontransfected cells to the indicated levels of IPTG (black lines). Each data point represents the mean expression of annexin V and PI in four independent experiments.

a particular gene, it only allows for the comparison of two extreme cases of gene expression, on versus off. Because our switch exhibits undetectable expression levels of the transgene in the off state and has the ability to tune the expression of the transgene, it will allow the design of experiments to study phenotypes based on threshold responses of gene expression both in vitro and in vivo. Along these lines, we used LTRi to control the expression of *bax*, a proapoptotic gene, in primary mouse cells and found a possible threshold response for Bax-induced apoptosis.

Using LTRi to regulate the expression of Cre in transgenic mice would give rise to an in vivo, nontoxic inducible knockout system that allows for controlling the timing of the specific gene to be knocked out. This will make it possible to study genes that would otherwise be lethal if removed too early in the development of the mouse. We have established the feasibility of this application by showing that LTRi can be used to tightly regulate the expression of Cre recombinase in primary mouse fibroblasts. Additionally, using LTRi to regulate *DTA* could be of significant value for a variety of mechanistic studies—e.g., neurodegenerative diseases could be studied by using a tissue-specific promoter to destroy a single type of neuron in mice. The ability to control the expression of *DTA* with LTRi could also be used for targeting cancer cells (Anderson et al., 2004; Peng et al., 2002). Overall, this work establishes a system for tight, tunable, reversible control of mammalian gene expression that can be used to explore a number of biological processes (e.g., stem cell differentiation) and the functional role of various genes (e.g., those involved in development or the onset of disease).

## EXPERIMENTAL PROCEDURES

### Synthetic Network Construction

The CMV, RSV, and U6 promoters were amplified by PCR from pCMVLacI (Stratagene), pOPI3CAT (Stratagene), and U6/tetO (a gift from D. Takai, University of Tokyo), respectively. The SV40 introns containing three *lac* operator sites were PCR amplified from pOPI3CAT (Stratagene). Genes encoding EGFP, TetR, and LacI were amplified by PCR from pIRES2-EGFP (Clontech), pcDNA6/TR (Invitrogen), and pCMVLacI (Stratagene), respectively. The genes encoding *DTA* and neomycin resistance were cut out of p22EDT1 (a gift from J. Sawicki, Lankenau Institute for Medical Research) and pNHS103\_v3 (a gift from M. Deans, Harvard Medical School) plasmids, respectively. The *bax* gene was PCR amplified from pcDNA3-*bax* (Addgene) and inserted into LTRi.

### Cell Culture Conditions and Transfections

CHO-K1 and HEK293 cells were obtained from ATCC (ATCC CCL61 and CRL1573, respectively). The CHO-K1 cells were maintained in F12K medium containing 10% FBS and penicillin/streptomycin, and the HEK293 cells were maintained in DMEM medium containing 10% FBS and penicillin/streptomycin. Both cells lines were grown in a humidified 5% CO<sub>2</sub>, 37°C incubator. The day before transfection, cells were plated in a 12-well plate to ~85% confluency. These cells were transfected with 1.6 µg of expression plasmid using Lipofectamine 2000 reagent (Invitrogen). For the transient experiments, cells

were washed with PBS, trypsinized, and analyzed by flow cytometry 24 hr after transfection.

Rosa26 fibroblasts were obtained from embryonic day 14.5 Rosa26-R mice. The cells were maintained in embryonic fibroblast medium (high-glucose DMEM, 10% FBS, penicillin/streptomycin, L-glutamine, and sodium pyruvate) and grown in a humidified 5% CO<sub>2</sub>, 37°C incubator. These cells were transfected with 0.8 µg of LTRi-*cre* and LTRi-*bax* using Lipofectamine 2000 reagent (Invitrogen). The LTRi-*cre* cells were analyzed by β-galactosidase staining 48 hr after transfection. The LTRi-*bax* cells were stained and analyzed by flow cytometry 48 hr after transfection.

For establishing stable cell lines, cells were plated in a 10 cm dish at ~85% confluency the day before transfection. The expression vector was linearized, and cells were transfected with the linearized vector using Lipofectamine 2000 per the manufacturer's protocol. Twenty-four hours after transfection, cells were passed and cultured in the presence of the antibiotic G418 (600 µg/ml, Invitrogen) for 2 weeks. The neomycin resistance (*neo*) gene encoded on the vector confers resistance to G418 and is an indication of stable integration of plasmid DNA into the host cell chromosomes. Stable colonies were picked and grown in separate dishes for analysis using flow cytometry.

### Inducing Cells with IPTG

For the transient experiments, 1 mM of IPTG was added shortly after the transfections were performed. For the stable experiments, fresh medium containing IPTG was added daily to the cells.

### Flow Cytometry

For the EGFP analysis, transfected cells were harvested, washed, and resuspended in PBS. Fluorescence data were collected using a FACSCalibur flow cytometer (BD Biosciences) integrated with CellQuest software. The cells were analyzed with a 488 nm argon excitation laser and a 515–545 nm emission filter (FL1). Data analysis was performed using WinMDI software, version 2.8 (J. Trotter, The Scripps Research Institute) and MATLAB (The MathWorks, Inc.). Cell samples were assayed at a medium flow rate until 30,000 cells had been collected within a forward-scatter and side-scatter gate to minimize fluorescence variation due to cell size.

For the apoptosis study, cells were stained with Alexa Fluor 488-conjugated annexin V and propidium iodide (PI) using the Vybrant Apoptosis Assay Kit (Invitrogen) per the manufacturer's protocol. Cell samples were assayed at a medium flow rate until 15,000 cells had been collected.

### Fluorescence Microscopy

Coverslips were treated with protamine and placed in the bottoms of a 12-well plate. Cells were grown on these coverslips overnight and transfected the next day with 1.6 µg of expression plasmid using Lipofectamine 2000 reagent (Invitrogen) per the manufacturer's protocol. The following morning, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After fixation, the coverslips were washed with PBS, and mounting medium was placed on the cells. The coverslips were then mounted face down onto slides, and the cells were visualized for fluorescence using a Nikon Eclipse 80i microscope.

### Staining for β-Galactosidase

Cells were grown in 12-well plates, and 24 hr after plating, they were transfected with 0.8 µg of LTRi-*cre* using Lipofectamine 2000 reagent (Invitrogen) per the manufacturer's protocol and exposed to 1 mM IPTG. Cells were incubated for 48 hr, fixed with paraformaldehyde, washed with PBS, and stained with X-gal staining buffer.

### Staining for Apoptosis and Cell Death

Cells were grown in 12-well plates, and 24 hr after plating, they were transfected with 0.8 µg of LTRi-*bax* using Lipofectamine 2000 reagent (Invitrogen) per the manufacturer's protocol and exposed to the indicated levels of IPTG. Cells were incubated for 48 hr, and adherent cells

were harvested by mild trypsinization and pooled together with detached cells. Cells were stained with Alexa Fluor 488-conjugated annexin V and PI using the Vybrant Apoptosis Assay Kit (Invitrogen) per the manufacturer's protocol. In live cells, phosphatidylserine is located in the cytoplasmic surface of the cell membrane; however, in apoptotic cells, it translocates to the outer leaflet of the plasma membrane (van Engeland et al., 1998). Annexin V has a high affinity for phosphatidylserine; therefore, annexin V conjugated with Alexa Fluor 488 can identify apoptotic cells by binding to phosphatidylserine and can be analyzed by flow cytometry measuring the fluorescence emission at 530 nm (FL1). PI accesses the inner cell of dead cells due to damaged plasma membranes and binds tightly to nucleic acids. It is impermeable to live and apoptotic cells but stains dead cells with red fluorescence. Dead cells appear red and can be analyzed by flow cytometry measuring the red fluorescence emission >575 nm (FL3). Moreover, live cells are annexin V negative and PI negative, apoptotic cells are annexin V positive and PI negative, and dead cells are annexin V negative and PI positive.

#### Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, and three figures and can be found with this article online at <http://www.cell.com/cgi/content/full/130/2/363/DC1/>.

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