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The REMOTE-control system: a system for reversible and tunable control of endogenous gene expression in mice

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ABSTRACT

We report here a robust, tunable, and reversible transcription control system for endogenous genes. The REMOTE-control system (Reversible Manipulation of Transcription at Endogenous loci) employs enhanced *lac* repression and *tet* activation systems. With this approach, we show in mouse embryonic stem cells that endogenous *Dnmt1* gene transcription could be up- or downregulated in a tunable, inducible, and reversible manner across nearly two orders of magnitude. Transcriptional repression of *Dnmt1* by REMOTE-control was potent enough to cause embryonic lethality in mice, reminiscent of a genetic knockout of *Dnmt1* and could substantially suppress intestinal polyp formation when applied to an Apc^{Min} model. Binding by the enhanced lac repressor was sufficiently tight to allow strong attenuation of transcriptional elongation, even at operators located many kilobases downstream of the transcription start site and to produce invariably tight repression of all of the strong viral/mammalian promoters tested. Our approach of targeting tet transcriptional activators to the endogenous *Dnmt1* promoter resulted in robust upregulation of this highly expressed housekeeping gene. Our system provides exquisite control of the level, timing, and cell-type specificity of endogenous gene expression, and the potency and versatility of the system will enable high resolution *in vivo* functional analyses.

INTRODUCTION

Genetic manipulation of mammals has been integral to the interrogation of gene function in a native biological context (1). Loss- or gain-of-function approaches by genetic knockout or transgenic technologies have long served as a direct, powerful means to reveal gene function. However, these dichotomous (on/off) and non-temporal approaches are not well-suited for understanding the full functional spectrum of a gene as the functional manifestation of a gene depends critically on spatio-temporal context and its distinct expression levels therein. Therefore, an ability to control the timing, location, and level of a gene expression would be ideal for precise functional characterization. Conditional knockout technologies have enabled spatio-temporal regulation, but limitations associated with their dichotomous nature, such as a cell lethality, continue to pose challenges and do not allow for studying the phenotypic consequences of variable levels of gene expression. Conditional knockdown approaches using *tet*-regulated shRNA or miRNA have helped to fill this void, but their successful implementation is not universal (2–4). Off-target effects remain an intrinsic problem for RNAi (5) and are difficult to control *in vivo*. CRISPR/Cas-mediated technologies have provided a new, versatile means of controlling endogenous gene expression and will greatly improve our study of gene function (6,7), although the efficacy of *in vivo* transcriptional control using CRISPR/Cas systems is unclear.

In principle, modification of endogenous gene promoters with binding sequences for transcriptional repressors or activators with regulatory ligands would allow for tunable, inducible and reversible gene expression control. Indeed, a few reports have described such exquisite control of endogenous gene expression by prokaryotic binary systems (8–11). However, the insufficient potency of the existing binary systems and the technical challenge associated with the modification of endogenous genes by conventional gene targeting have hampered their broad application. Recently, the latter challenge has been substantially mitigated by the introduction of CRISPR/Cas-mediated genome editing technologies, with which endogenous gene modification has now become a routine task (12–16).

Here, we provide (i) an engineered binary system sufficiently potent for endogenous gene repression, (ii) a first proof of principle for the inducible transcriptional upregulation of an endogenous gene from its cognate promoter in

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mice and (iii) a standardized and efficient application strategy.

We have engineered an enhanced *lacI* repression system that is substantially stronger than the wild-type *lac* system (>100 times) and produces consistently tight repression of all of the widely used, strong viral/mammalian promoters we tested. We also show that tight repression can be achieved even through inhibition of transcription elongation from operators located in an intron far downstream of transcription start sites, providing a simple strategy for repression without affecting the endogenous promoter. Moreover, we were able to upregulate expression 6.5-fold from an already highly expressed gene, *Dnmt1*, by targeting *tet* activators to its endogenous promoter. The availability of efficient regulatory ligands for both the *lac* repressor (IPTG) and the *tet* activator (doxycycline) makes the REMOTE-control system tunable and reversible (Figure 1A), providing exquisite versatility for high resolution *in vivo* functional analyses. The system could be expanded to other organisms and binary systems.

MATERIALS AND METHODS

Luciferase reporter assay

NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (tetracycline-free, Hyclone) and 1% penicillin/streptomycin. Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used for transfection. The Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) was used to determine luciferase expression. The ratio of firefly to renilla luminescence intensity was calculated and used as a measure for promoter activity.

Gene-targeting

The *Dnmt1LO* and *LGT* alleles were generated using a conventional gene-targeting procedure. ES cells were maintained in HEPES-buffered (20 mM [pH 7.3]) DMEM supplemented with 15% fetal calf serum (GE Heathcare Life Sciences, Pittsburgh, PA, USA), 0.1 mM nonessential amino acids (Thermo Fisher Scientific, Waltham, MA, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and penicillin–streptomycin (Thermo Fisher Scientific, Waltham, MA). ES cells were grown on feeder layers of gamma-irradiated embryonic fibroblast cells and supplemented with leukemia inhibitory factor (LIF) (Thermo Fisher Scientific, Waltham, MA) at 10^6 U/ml. ES cells were electroporated with 20 μg of linearized DNA in PBS at a set voltage 230 V and a capacitance of 500 μF with a Bio-Rad GenePulser II. Antibiotic selection was initiated the following day and continued for 8–11 days before picking. G418 was applied to the culture medium at a concentration of 300 μg/ml. PCR screening was performed using EXL DNA polymerase (Agilent Technologies, Santa Clara, CA, USA) with following primers; 5′-TGCATCCTAGGCCTATTAATATTC-3′ (forward) and 5′-GAAGTGACTGTCACACAAGCACA-3′ (reverse). Cre recombinase was introduced by electroporation as described above. Ganciclovir (510 ng/ml) selection was performed to screen for the TkNeo removed ES cells. PCR screening was performed with following primers; 5′-GGTTCAGAAGGGCCCTCCTT-3′ (forward) and 5′-CCAGGCAGAGTCTCTTTGATAG-3′ (reverse).

RT-PCR assays

Total RNA was prepared using TRIzol (Thermo Fisher Scientific, Waltham, MA). cDNAs were made using Superscript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA), as per the manufacturer's instructions, and amplified by PCR with Taq polymerase. See Supplementary Materials and Methods for additional details.

Gene expression array

Eight RNA samples were analyzed using the Illumina MouseRef-8 v2.0 Expression BeadChip array (Illumina) at the University of Southern California Epigenome Center. The quality of the RNA samples was assessed using spectrophotometry and capillary electrophoresis. All samples were quantified on an ND-8000 (ThermoScientific). The samples were then run on Experion RNA StdSens Analysis chip (Bio-rad) to ensure that the RNA was of sufficient quality, free from DNA contamination and not overly degraded. All samples that passed the initial QC checks were processed using TotalPrep RNA Amplification Kit (Ambion). First, 500 ng of RNA from each sample was converted using reverse transcription to single-stranded cDNA. Next, the second cDNA strand was synthesized, and the resulting double-stranded cDNA was purified. This cDNA was then converted to biotin-labeled cRNA through an overnight *in vitro* transcription step. The cRNA was then purified using the provided filter columns and was quantified by spectrophotometry using the ND-8000. The cRNA samples were brought to a concentration of 150 ng/μl. cRNA (750 ng) was mixed with a hybridization buffer and added to a MouseRef-8 v2.0 Expression BeadChip (Illumina). The samples were allowed to incubate overnight on the chips and were then stained and scanned using an Illumina HD Beadarray scanner. Distribution of densities and signal intensities were highly similar between all samples. Data underwent $\log_2$ transformation and quantile normalization using Partek Genomics Suite 6.5 software (Partek). Fold changes were calculated from the average signal intensity from the normalized Data. Data from gene expression array experiments have been deposited in GEO (accession number: GSE28386).

Statistics

All quantitative data were collected from experiments performed in at least triplicate, and expressed as mean ± standard Error. All experiments, except the ones in Figure 5C, D, and Supplementary Figure S7D, were analyzed in Prism as follows: differences between two groups were assayed by two-tailed unpaired *t*-tests and differences between more than two groups were assayed by one-way ANOVA, where Dunnett's *t*-tests were used to determine which groups (if any) differed significantly from the control. The remaining experiments were assayed in R 3.2.0 via ANOVA, but

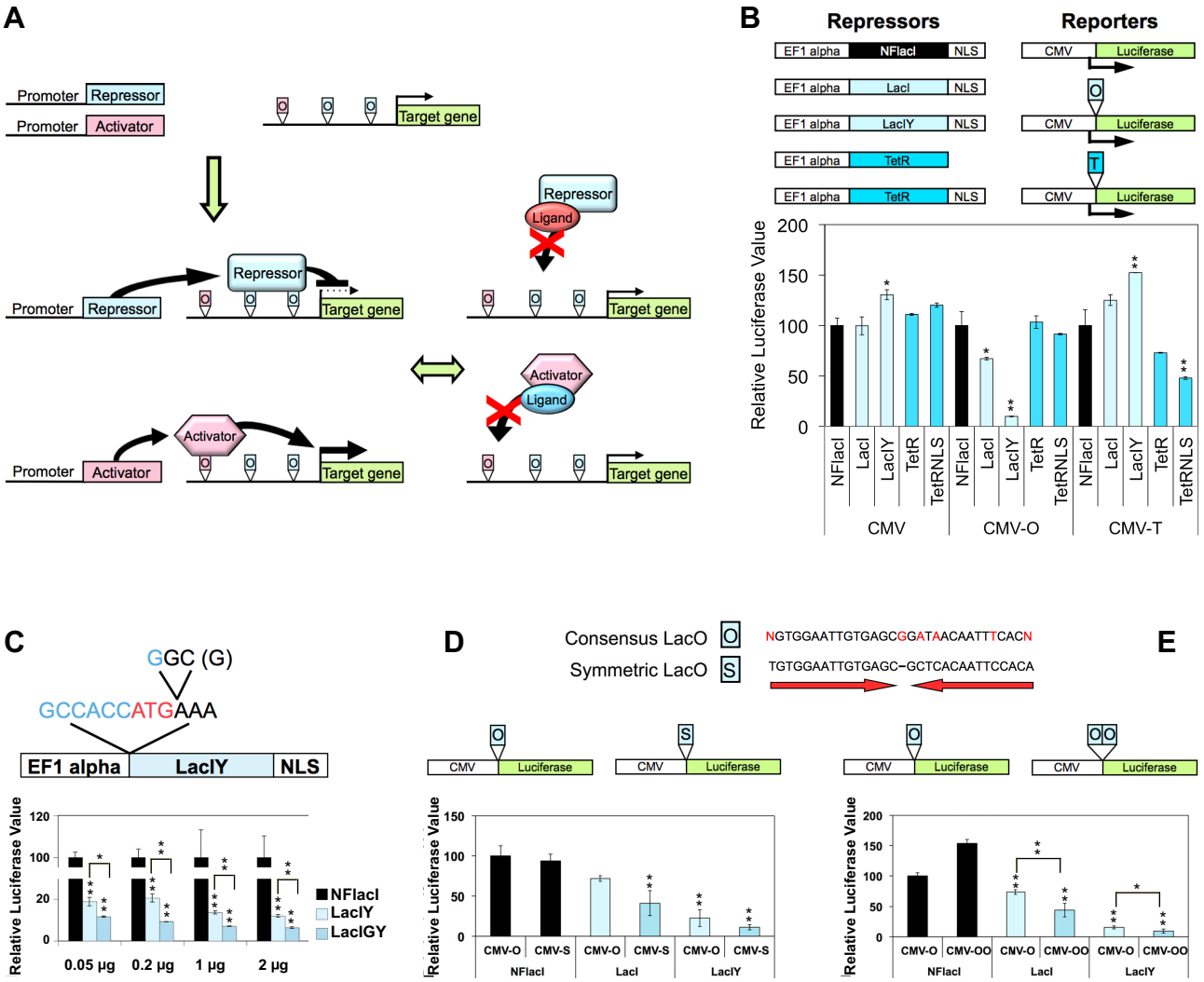

Figure 1. *In Vitro* Optimization of the REMOTE-control System. (**A**) Schematic view of the REMOTE-control system. The transcription of a target gene can be up- or downregulated upon the binding of activators or repressors to the corresponding operators. Regulatory ligands of the repressor and transactivator reverse the transcriptional regulations. (**B**) Repressor and reporter constructs used in the transient reporter assay: non-functional *lacI* (*NFlacI*); wild-type *lacI* (*lacI*); *lacI* with a tight-binding mutation (*lacIY*); wild-type *tet* repressor (*TetR*); *tet* repressor with a nuclear localization signal (*tetR*NLS); *lac* operator (O); and *tet* operator (T). CMV-O and CMV-T refer to the reporter containing a *lac*O or a *tet*O, respectively. Reporters (50 ng/well in 96-well plate) and corresponding repressors were co-transfected into NIH/3T3 cells in a 1:1 molar ratio and assayed 24 h after transfection (one-way ANOVA). See also Supplementary Figure S1A–C. (**C**) Top: *lacIY* with a stabilizing amino acid insertion; the Kozak sequence is indicated in blue. Bottom: Reporter assays for *lac* repressors containing the stabilizing amino acid. Experiments were performed in 24-well plates. Reporter plasmid (2 µg/well) was co-transfected with four different amounts of repressor plasmids as indicated. Luciferase activity was measured 48 h after transfection (between two groups: *t*-tests, between more than two groups: one-way ANOVA). (**D**) Sequence of the consensus (O) and symmetric *lac* operators (S). The reporter (50 ng/well in 96-well plate) harboring either the consensus or symmetric operator was co-transfected with repressor as indicated (between two groups: *t*-tests, between more than two groups: one-way ANOVA). (**E**) The reporter containing either one or two operators (50 ng/well in 96-well plate) was co-transfected with repressor as indicated (between two groups: *t*-tests, between more than two groups: one-way ANOVA). Luciferase data are presented as percent relative to the reference value set to 100%. Data are represented as mean ± SEM ($n = 3$). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

instead of Dunnett's *t*-tests, false discovery rate corrected Welch's *t*-tests were used to determine which groups differed significantly from the control; this method was chosen to appropriately account for the strong heteroskedasticity present in these data. Regardless of software, significant differences were considered when $P < 0.05$; $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

RESULTS

In vitro optimization of the REMOTE-control system

The first step in developing an effective transcriptional control system capable of both activation and repression is to evaluate which system is best suited for each task. We focused our efforts on the *lac* and *tet* systems as two of the best characterized and most widely used reversible binary transcriptional regulatory systems. To test their repressive capabilities, we constructed reporters with a single-copy *lac*

operator (*lac*O) or *tet* operator (*tet*O) placed between the CMV promoter and the luciferase gene. All repressor constructs were engineered to have identical plasmid backbones and promoters to ensure a fair comparison of their repressive capacity per gene copy. Given differences in translation efficiency and protein stability (see below), that measure is more appropriate for transgenic applications than a comparison of repressor proteins on an equimolar basis (Figure 1B).

We performed the comparison with five different repressors: a *lac* repressor with codon usage optimized for mammalian cells, producing a wild-type repressor protein (17) (referred to as *lacI*); a *lac* repressor with a tight-binding mutation (*lacIY*); a non-functional *lac* repressor control (*NFlacI*); a wild-type *tet* repressor (*TetR*); and a *tet* repressor with nuclear localization signal (*TetRNLS*). We generated *lacIY* by adopting a tight-binding mutation identified in *E. coli* that increases the repressor-to-operator binding affinity ∼100-fold (18). We engineered *tetRNLS* by inserting the nuclear localization signal used for the *lac* repressors into the *tet* repressor.

The *lac*O or *tet*O insertion alone had little effect on the promoter in the absence of repressors (Supplementary Figure S1A). The reporter and repressor plasmids were transiently introduced into NIH/3T3 cells at a 1:1 plasmid molar ratio. Among the repressors, the tight-binding mutant *lacIY* showed the strongest repression of the CMV promoter (a 90% reduction of luminescence relative to *NFlacI*), while other repressors showed modest repression (Figure 1B). To exclude possible biases, including the fact that *lac* and *tet* repressors were tested on two different reporters separately, we also used reporters containing both operators in tandem in both sequential configurations, and with repressors introduced at various concentrations (up to an 8-fold molar excess) (Supplementary Figure S1B and C). For this comparison, we used *lacIY* and *tetRNLS* as the best-performing repressors for each system. We found that *lacIY* repressed the CMV promoter substantially better under all experimental conditions (Supplementary Figure S1B and C). Based on the level of repression and the less stringent constraints on the position of its operator, we chose the *lac* operator/repressor system as the repression module for the REMOTE-control system.

A lysine residue at the N-terminus of a protein is a primary destabilizing amino acid in mammals (19). We therefore inserted a stabilizing amino acid into the *lacIY* repressor to mask its N-terminal lysine. We chose glycine, since it is one of the four stabilizing amino acids in mice (19) and because its optimal codon in mammals (GGC) adds a critical component of the Kozak consensus eukaryotic translation initiation sequence (guanine at +4) (20) when placed immediately adjacent to the initiation codon (Figure 1C). This glycine insertion in *lacIY*, giving *lacIGY*, resulted in improved repression at four different repressor-to-reporter ratios (Figure 1C).

The consensus *lac* operator is an imperfect palindromic 29-bp sequence with just four mispairs (Figure 1D). A 30-bp perfectly symmetric *lac* operator sequence interacts with the *lac* repressor 8–10 times more strongly than the consensus sequence (21). We used this modified *lac* operator to further improve operator/repressor binding affinity. The reporter having the symmetric operator sequence was repressed more strongly by both *lacI* and *lacIY* than the reporter having a consensus operator (Figure 1D). Finally, we were able to further improve repression by about 50% by simply doubling the number of operator sequences (Figure 1E).

Modification of the *Dnmt1* promoter

Homozygous knockout of the *Dnmt1* gene generally results in cell death, interfering with our ability to use conditional knockouts to study *Dnmt1* deficiency *in vivo* (22). Moreover, *Dnmt1* is a highly expressed housekeeping gene, and robust overexpression of the full-length protein has been difficult to achieve in cell culture (23) and has resulted in embryonic lethality *in vivo* (24). Thus, this gene was a good candidate for testing our REMOTE-control system.

We first defined the minimally effective *Dnmt1* somatic promoter in the mouse (25). A 281-bp fragment (pDL5) spanning the transcription start site (–182 to +99) conferred full expression potential in NIH/3T3 cells as previously reported (26) (Data not shown). We next selected candidate operator insertion sites within this 281-bp fragment containing the *Dnmt1* promoter that avoided disrupting evolutionarily conserved sequences or putative transcription factor binding sites obtained from a screen for consensus binding sites and from functional studies (Figure 2A) (26,27). We also included a candidate insertion site in the 5′ UTR (+99), as we had observed efficient repression from an operator located downstream of transcription start site of the CMV promoter (Figure 1B, Supplementary Figure S1B and C). We devised a PCR-based cloning method using a shared set of just three restriction enzymes for the promoter deletions and operator insertions, thus streamlining the construction of plasmids with multiple operator insertions at any desired location (Supplementary Figure S2A). This method is in principle applicable to any promoter.

We inserted *lac*O sequences at the selected sites and assessed their effects on promoter efficacy in the absence of repressor. Insertions at –182, –59 and +99 (pDL19) resulted in a promoter strength comparable to that of the unmodified promoter (Figure 2A). We then tested the symmetric operator and double-operator sequences at –182, –59 and +99. Location –59 could tolerate only a single operator (data not shown), but location +99 could accommodate a double consensus operator without a noticeable effect on promoter efficacy (data not shown). However, the double symmetric operator severely suppressed luciferase activity when located immediately downstream of the transcription start site of any promoter construct in the absence of the *lac* repressor (data not shown). This is likely attributable to the formation of strong hairpin structures in the 5′ UTR of the mature mRNA, since we did not observe this effect of double symmetric operators when they were in an intron (Figure 6A, Supplementary Figure S7A). In summary, we inserted a double symmetric operator at –182, a single symmetric operator at –59 and a double consensus operator at +99. We further modified the *Dnmt1* promoter with *tet* operators and *gal4* DNA binding sequences (DBS) at –182 to achieve upregulation using the *tetVP16* (*tTA*) and *gal4VP16* transactivators.

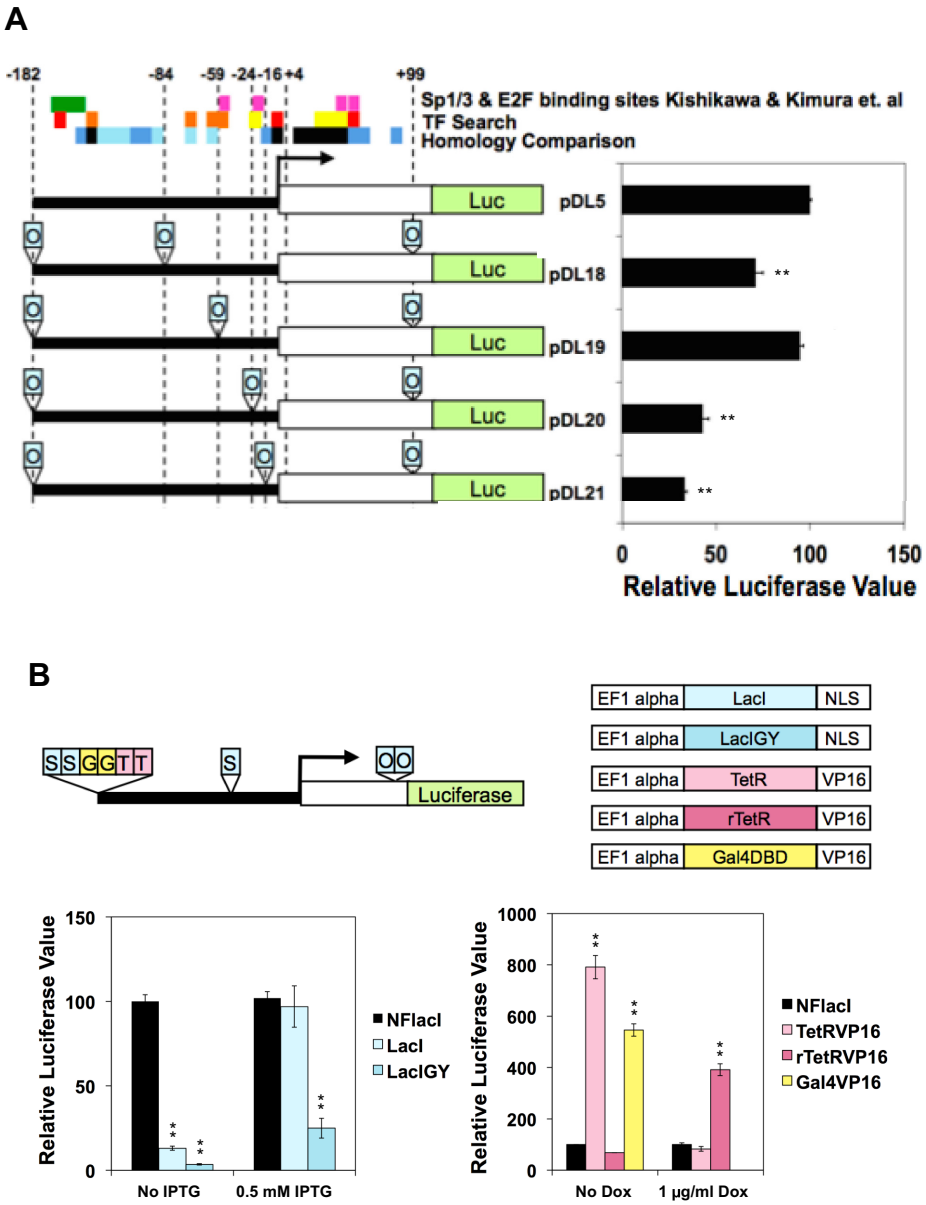

Figure 2. Applying the System to the *Dnmt1* Promoter. (**A**) Reporter assay with the *Dnmt1* promoters modified with *lac* operator (O) sequences in the absence of the *lac* repressor. The 281-bp sequence immediately upstream of the somatic transcription start site was subjected to a transcription factor binding site search (TFSEARCH: http://www.cbrc.jp/research/db/TFSEARCH.html) and to homology comparison with rat and human *DNMT1* promoter sequences. Putative transcription binding sites are color coded from red to light yellow according to their scores (100–96, red; 95–91, orange; 90–85, yellow). Regions with high homology are shown as black. Green and pink rectangles represent Sp1/3 and E2F binding sites, respectively (one-way ANOVA). (**B**) The reporter containing the modified *Dnmt1* promoter and repressors were transfected in the presence or absence of IPTG (left panel). See Supplementary Figure S2C for higher amounts of IPTG treatment. The reporter and transactivators were transfected in the presence or absence of doxycycline (right panel) (one-way ANOVA). Luciferase data are presented as percent relative to the reference value set to 100%. Data are represented as mean ± SEM ($n = 3$). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

Up- and downregulation of the *Dnmt1* promoter by the REMOTE-control system *in vitro*

We first tested whether the *lac* repressor could repress the modified *Dnmt1* promoter by introducing the *lacI* or *lacIGY* plasmids into NIH/3T3 cells together with the reporter plasmid containing the modified *Dnmt1* promoter. Both the wild-type and modified *lac* repressors successfully repressed transcription from the *Dnmt1* promoter with operator insertions (Figure 2B). Almost complete repression (3.7% residual expression) was achieved with *lacIGY*. The repression by *lacI* was completely reversed by 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), while that treatment restored only 25% of unregulated expression for *lacIGY*. We also tested whether repression by *lacIGY* could be completely reversed by molar concentrations of IPTG from 0.5 mM to 10 mM. Even the best restoration, at 3 mM IPTG, gave only 40% of unregulated expression (Supplementary Figure S2B). However, subsequent experiments in stably

transfected single-copy cell lines and in mice showed almost complete derepression of *lacIGY* by IPTG (Figure 4D and E, Supplementary Figure S6B). This suggests that the incomplete reversion in transient transfection experiments might be due to the high reporter plasmid copy number and large amounts of lacIGY in transiently transfected cells.

To achieve upregulation of the *Dnmt1* promoter, we introduced the *tetVP16* or *gal4VP16* transactivator plasmids into NIH/3T3 cells. In transient reporter assays, we observed 5-fold and 8-fold inductions for *gal4VP16* and *tetVP16*, respectively, and the 8-fold induction by *tetVP16* was completely abolished by doxycycline treatment, an antagonist of *tet* (Figure 2B). We also tested a reverse version of *tetVP16*, *rtetVP16* (*rtTA*), which binds to the operator when doxycycline is bound to achieve inducible upregulation. With 1 μg/ml of doxycycline, *rtetVP16* increased the expression by 4-fold, and no induction was observed in the absence of doxycycline (Figure 2B). With these three modifications together, we could achieve repression of the Dnmt1 promoter down to 0.03-fold and upregulation up to 8-fold. We chose the *tet* system over the *gal4* system for our upregulation approach, owing to its reversibility, but in principle, the gal4 system could be used to add additional controls to the *Dnmt1* expression regulation by recruitment of other transcription regulators, such as histone modifying enzymes or transcription factors.

Applying the REMOTE-control system to the endogenous *Dnmt1* promoter and testing the repression system in ES cells

We identified three locations at which to insert *lac* operators without affecting the activity of the minimally effective *Dnmt1* promoter (–182 to +99). We used homologous recombination in mouse embryonic stem cells to introduce *lac* operators at the endogenous *Dnmt1* promoter to generate the modified *Dnmt1* allele designated $Dnmt1^{LO}$ (Figure 3A). We stably introduced *lac* repressor constructs into the targeted ES cells to test repression efficacy in the context of a native chromatin configuration prior to testing the system in mice. We developed a real-time RT-PCR reaction specific for the $Dnmt1^{LO}$ allele to analyze the level of repression of the targeted allele (Figure 3B). We performed expression analysis with RNA samples from several independent clones of each of the ES cell lines. We observed ∼60% transcriptional repression with wild-type *lacI*, but in excess of 90% repression in five out of six ES cell clones expressing *lacIY* (Figure 3B). The *lacI* and *lacIY* clones showed comparable levels of expression (Supplementary Figure S3A and B), so we infer that the difference in repression efficacy is likely due to binding affinity differences between the two repressor proteins.

In vivo downregulation of *Dnmt1* expression by the *Lac* system

We introduced the $Dnmt1^{LO}$ modification into the mouse germline and generated $Dnmt1^{LO/LO}$ homozygous mice (Figure 4A). We did not observe any difference in *Dnmt1* expression in the colon between $Dnmt1^{+/+}$ and $Dnmt1^{LO/LO}$ mice in the absence of *lac* repressor (Figure 4C), confirming that our *lac*O modification had not disrupted normal promoter function. We next developed tissue-specific and ubiquitous transgenic mouse lines for the *lacIGY* (Figure 4A). We generated intestine-specific lines using the *Villin* promoter, which restricts the expression of a transgene to the intestine, colon and kidney proximal tubules (28) (Figure 4A and B). Among the three transgenic lines we developed, line 3 showed a robust, intestine-specific expression of *lacIGY*. We crossed this transgenic line with the $Dnmt1^{LO}$ mice and generated $Dnmt1^{LO/LO}/Tg^{VillacIGY}$ mice to test the *in vivo* efficacy of transcriptional repression. $Dnmt1^{LO/LO}/Tg^{VillacIGY}$ mice reduced *Dnmt1* expression to 15% of the levels observed in $Dnmt1^{LO/LO}$ mice without the repressor transgene (Figure 4C). These results show for the first time that the *lac* repressor can inhibit transcription of an endogenous gene at its cognate locus in mice. We confirmed the reduction in *Dnmt1* expression at the protein level by immunostaining. Nuclear staining of Dnmt1 protein disappeared almost completely from colonic crypts of $Dnmt1^{LO/LO}/Tg^{VillacIGY}$ mice (Figure 4D).

We next investigated whether repression by the *lac* repressor could be reversed by IPTG treatment. Previous studies showed that IPTG can be efficiently applied to various mouse tissues via oral administration or intravenous injection and can effectively inhibit *lac* repression of exogenous transgenes in mice (17). We added IPTG to the drinking water for 3 weeks at concentrations of 20, 80 or 160 mM. Repression was reversed in a dose-dependent manner (Figure 4E). In contrast to the partial reversion in the transient transfection experiments (Figure 2B, Supplementary Figure S2B), expression *in vivo* was restored to 90% of unregulated expression at 160 mM IPTG (Figure 4E). Extended IPTG treatment for up to 7 months did not result in any notable deleterious effects. In addition, IPTG withdrawal resumed the tight repression of *Dnmt1* in 2–3 days (Supplementary Figure S4A).

We also tested the repression system in a broader spectrum of tissues using a ubiquitously expressing *lacIGY* line, with expression driven by the human elongation factor 1 alpha (*EF1α*) promoter (Figure 4A). One of the transgenic lines (line 11) exhibited high *lacIGY* expression across all the organs analyzed (Figure 4F). We observed that ubiquitous downregulation of *Dnmt1* by the *lac* system resulted in embryonic lethality (Chi-square test, $P < 0.0001$, Figure 4G). A genetically modified *Dnmt1* hypomorphic line having 10% of the expression of wild-type mice is viable (29). From this, it can be surmised that the level of residual *Dnmt1* expression in $Dnmt1^{LO/LO}/Tg^{EFlacIGY}$ is likely <10% in at least some essential tissues, impairing proper development. Expression analysis from mice heterozygous for the $Dnmt1^{LO}$ targeted allele showed that in the presence of *lacIGY,* transcription was reduced to approximately half-normal levels, consistent with almost complete repression of the $Dnmt1^{LO}$ allele, with the residual transcription from the remaining wild-type allele (Figure 4H).

DNA methylation and transcriptome analyses showed both global and gene-specific DNA methylation and gene expression changes upon the inhibition of *Dnmt1* (Supplementary Figure S5A-G, Tables S1–S3). This provides further support for the potency of our enhanced repression system, since trace amounts of Dnmt1 are sufficient to maintain substantial levels of genomic DNA methylation in cell

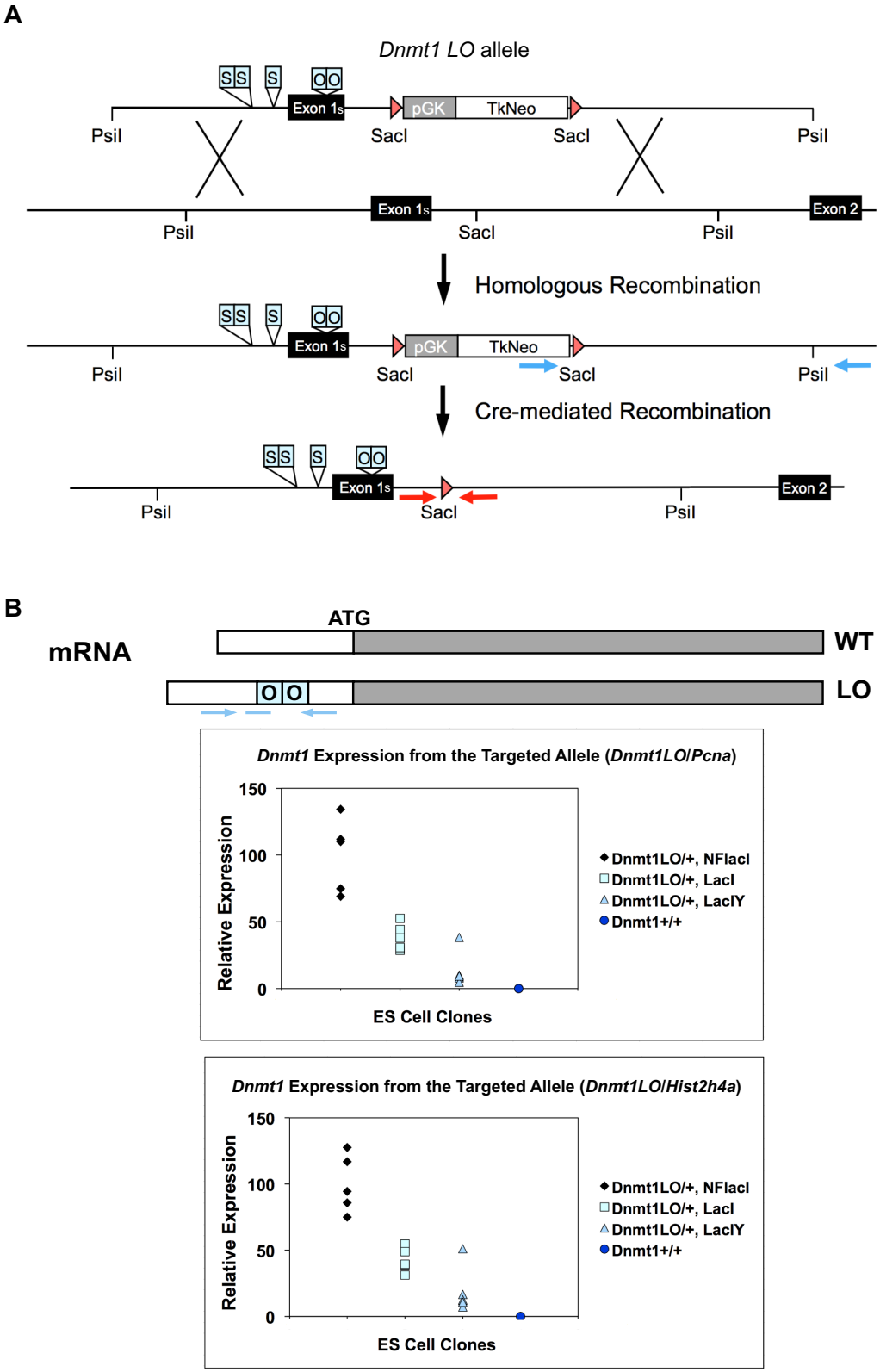

Figure 3. Modifying the endogenous *Dnmt1* promoter and repression of the endogenous *Dnmt1* by *lac* repressors in ES cells. (**A**) Gene-targeting was performed using a replacement-type vector containing the *Dnmt1* promoter modified with *lac* operator sequences as depicted. Blue arrows indicate the primers used in PCR screening after G418 selection. The selectable marker was removed from the targeted ES cells using Cre recombinase. PCR screening was performed across the single *loxP* generated as a result of the recombination that is unique to the targeted allele using primers shown as red arrows after ganciclovir selection. (**B**) *NFlacI*, *lacI*, and *lacIY* were stably introduced into the targeted ES cells through electroporation followed by hygromycin selection. Independent clones from each ES cell line were subjected to the real-time RT-PCR assays using a reaction specific for the targeted allele as indicated by blue arrows (primers) and line (probe). *Dnmt1*^{LO} expression was normalized against *Pcna* (left panel) or against histone *H4* (*Hist2h4a*) (Right panel). Expression levels of *lacI* and *lacIGY* are shown in Supplementary Figure S3.

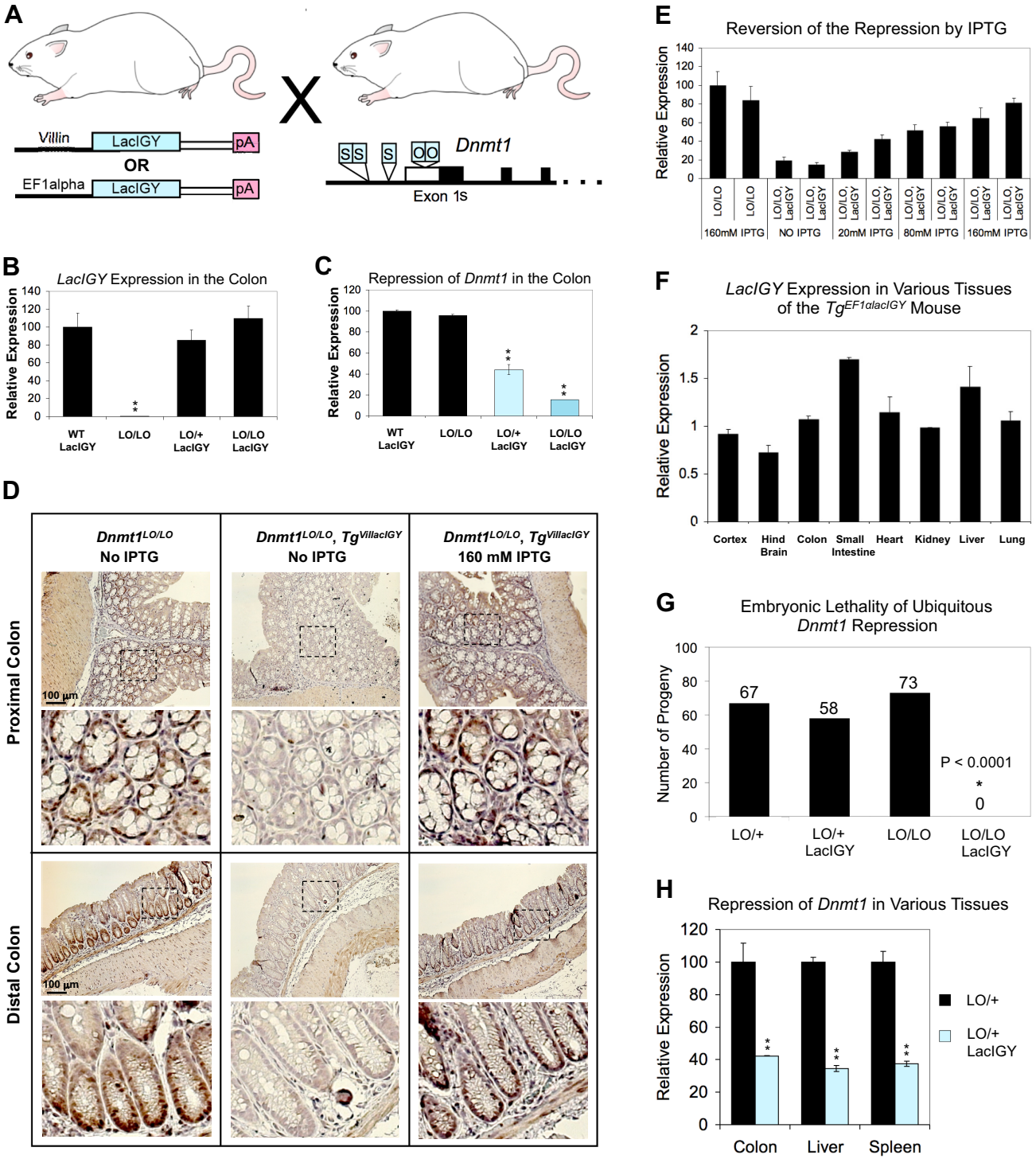

Figure 4. *In vivo* downregulation of *Dnmt1*. (**A**) The *lacIGY* transgenic (left) and $Dnmt1^{LO}$ (right) mice. The promoters driving *lacIGY* expression are indicated before the transgene. White thin boxes indicate the rabbit β-globin intron. (**B**) *LacIGY* expression from the colon (one-way-ANOVA). (**C**) *Dnmt1* expression in the colon from mice with or without the targeted allele ($Dnmt1^{LO}$) and *lacIGY*, using a real-time RT-PCR reaction designed to detect both the wild-type and targeted *Dnmt1* alleles (one-way ANOVA). (**D**) Immunostaining of Dnmt1 protein from the colon with and without IPTG treatment. For three weeks, mice were given IPTG-containing water kept in a light-protected bottle. (**E**) Real-time RT-PCR analysis showing the *in vivo* reversion of the repression from IPTG treatment. (**F**) *lacIGY* expression normalized by *Xist* in tissues from the *EFlacIGY* transgenic line. (**G**) Genotyping results from the $Dnmt1^{LO/LO} \times Dnmt1^{LO/+}/Tg^{EFlacIGY}$ cross, demonstrating the embryonic lethal phenotype *of* $Dnmt1^{LO/LO}$, $Tg^{EFlacIGY}$ mice. Genotyping was performed at birth (Chi-square test). (**H**) *Dnmt1* expression from the targeted allele ($Dnmt1^{LO}$) in tissues of $Dnmt1^{LO/+}/Tg^{EFlacIGY}$ mice (*t*-tests). Data are represented as mean ± SEM ($n = 3$). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$

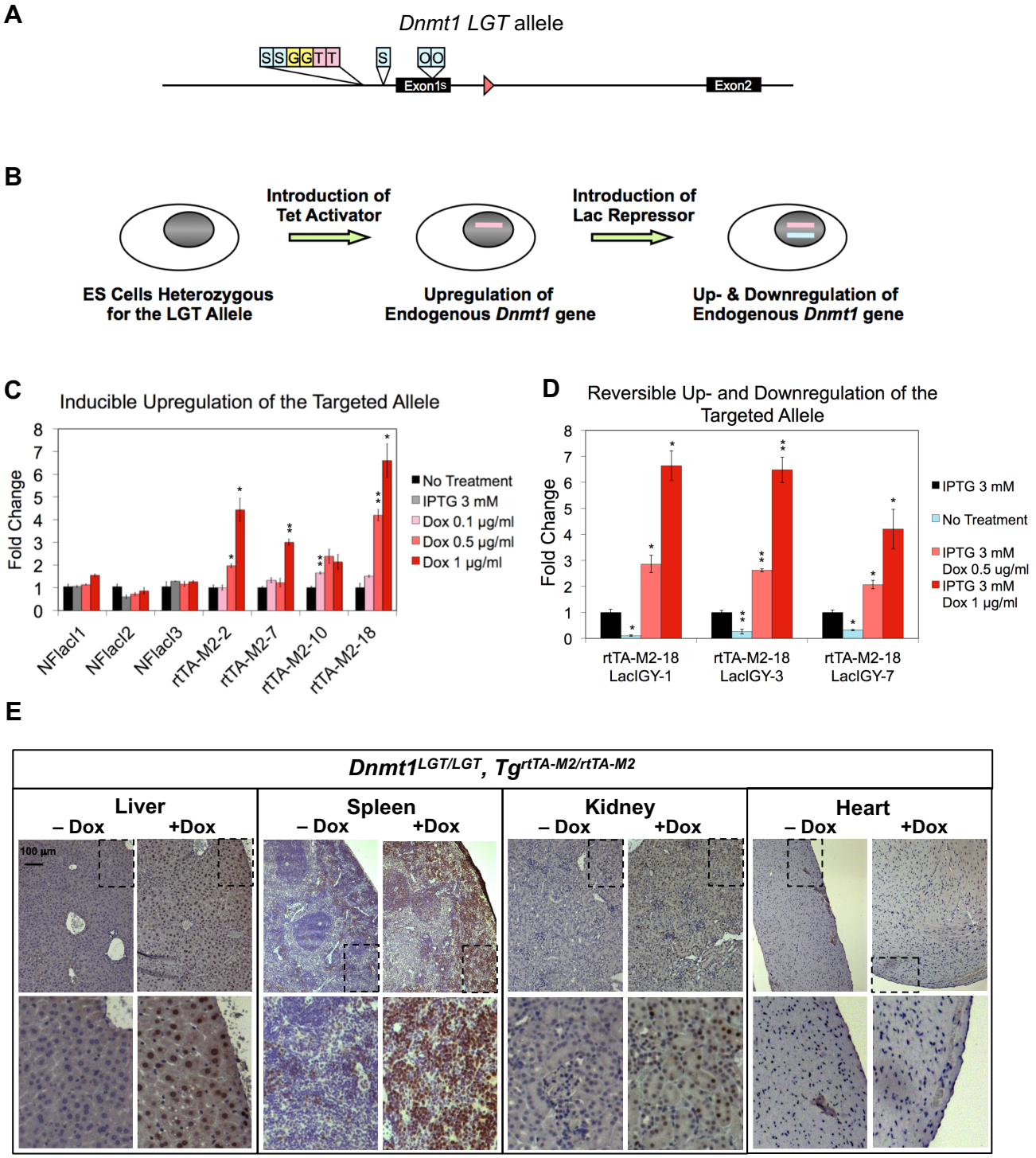

Figure 5. Up- and downregulation of endogenous *Dnmt1* expression. (**A**) Gene-targeting and screening were performed as described in Figure 3. (**B**) *NFlacI* or *rtTA-M2* was stably introduced into the targeted ES cells through electroporation followed by G418 selection. *lacIGY* was stably introduced into the *rtTA-M2*-expressing ES cells via electroporation followed by puromycin selection. Pink and blue lines indicate the integrated *rtTA-M2* and *lacIGY* transgenes, respectively. (**C**) $Dnmt1^{LGT}$ expression in the presence of IPTG or different doses of doxycycline. ES cell clones were subjected to real-time RT-PCR assays using a reaction specific for the targeted allele. (**D**) Up- and downregulation of the $Dnmt1^{LGT}$ allele by *lacIGY* and *rtTA-M2*. *Pcna* was used for normalization in all experiments. (**E**) Immunostaining results of Dnmt1 protein from various tissues with and without doxycycline treatment. Mice were treated with a doxycycline diet for one month. Data are represented as mean ± SEM ($n = 3$). $^*P \leq 0.05$, $^{**}P \leq 0.01$ (Welch's *t*-tests, see Methods for additional details).

lines and mice (30,31). As a surrogate for estimation of global methylation, we analyzed mouse repetitive element B1, B2 and IAP (Supplementary Figure S5A and B).

Inducible and reversible Up- and downregulation of endogenous *Dnmt1*

It has been particularly challenging to achieve strong overexpression of full-length *Dnmt1 in vitro* and *in vivo* (23,24). A 2.5-fold overexpression of *Dnmt1* from a *Dnmt1* BAC clone was found to result in embryonic lethality (24). No transgenic lines of *Dnmt1* have been reported, despite numerous attempts. Here, we report a new approach to achieve upregulation of *Dnmt1* by targeting transcriptional transactivators to the endogenous *Dnmt1* promoter. We combined two copies each of *tet* operators and of *gal4* DNA binding sequences with the *lac* operator insertions described above to create a $Dnmt1^{LGT}$ allele with more flexible control, i.e. allowing either upregulation or repression of transcription depending on which transacting protein is present (Figure 5A).

To test whether endogenous *Dnmt1* expression can be upregulated from the $Dnmt1^{LGT}$ allele, we stably introduced an advanced version of the inducible *tet* activator (*rtTA-M2*) into the targeted ES cells (Figure 5B). In the absence of doxycycline, *Dnmt1* expression from the *rtTA-M2*-expressing cells was comparable to that from control ES cells, but upon treatment with doxycycline, *Dnmt1* expression was increased up to 6.5-fold (Figure 5C and D, Supplementary Figure S6A and B). We confirmed that the $Dnmt1^{LGT}$ allele could also be downregulated 70–90% by introduction of the *lacIGY* repressor (Figure 5D). Thus, we were able to achieve either up- or downregulation of an endogenous allele in the same cells, using doxycycline to control activator binding and IPTG to reverse repressor binding.

We next introduced the $Dnmt1^{LGT}$ modification into the mouse germline and generated $Dnmt1^{LGT/LGT}$ homozygous mice. As we found from the colon, the modifications have no deleterious effect on the activity the endogenous *Dnmt1* promoter (Figure 4C, Supplementary Figure S6C and D). We crossed them with the available *ROSA26-rtTA-M2* transgenic line to test *in vivo* upregulation of *Dnmt1* (32). We observed a strong upregulation of *Dnmt1* from the liver, spleen, and kidney (Figure 5E). This provides the first experimental evidence that endogenous gene expression in mice can be upregulated from its cognate promoter by targeted recruitment of a transcriptional activator. We found no detectable upregulation in the heart, which could be explained by the proliferation-dependent expression pattern of *Dnmt1* and the sparse population of proliferation-competent cells in the heart (33) (Figure 5E). Thus, the stimulatory effects of *rtTA-M2* on transcription can enhance expression of a transcriptionally competent gene, but may not be sufficient to turn on a transcriptionally silent gene. This inability may be overcome by increasing the number of activator binding sites and the expression level of activator.

Transcriptional repression by attenuation of transcription elongation

Attenuation of transcription elongation by the *lac* repressor has been controversial. While successful blockage of transcription elongation has been reported (34), elongation factor SII enables RNA polymerase II to proceed through DNA-bound *lac* repressors at high efficiency (35). We hypothesized that the enhanced binding affinity of our modified *lac* system might be strong enough to overcome the effects of elongation factor SII. We tested this by inserting operators into the rabbit β-globin intron under the control of the *Dnmt1* promoter.

We observed moderate repression at intronic operators with the wild-type *lac* and *tet* repressors (Figure 6A, Supplementary Figure S7A), but the enhanced *lac* repressor indeed resulted in strong transcriptional repression (Figure 6A–D, Supplementary Figure S7A and B). We found that attenuation of transcription at downstream operators could be readily achieved with other strong promoters, including CMV, *Ubc* and even *EF1*α, one of the strongest mammalian promoters (Supplementary Figure S7B). We found no correlation between the level of residual expression and the strength of the promoters, suggesting that the repression capacity of our enhanced *lacI* system exceeds the transcriptional strength of all of the robust promoters we tested (Figure 6B, Supplementary Figure S7B and C).

In order to determine the *in vivo* efficacy of transcriptional attenuation by the REMOTE-control system, we generated a transgenic line with a reporter gene containing the *lac*O inserted rabbit β-globin intron and crossed with the $Tg^{VillacIGY}$ mice (Figure 6C). We observed more than 90% repression of the reporter in the small intestine (Figure 6C and D). This successful repression by inhibiting transcription elongation underscores the strength of the lacIGY binding, and further demonstrates the utility of our system. Furthermore, this method enables rapid application of the REMOTE-control system by simplifying the placement of operator sequences without disrupting normal expression in the absence of repressor, as introns contain far fewer binding sites for transcription factors than do promoters. This approach could also be advantageous for controlling genes with multiple promoters.

Suppression of intestinal polyp formation through repression of *Dnmt1* by the REMOTE-control system

Mouse models having lower *Dnmt1* expression have significantly fewer intestinal tumors induced by genetic mutations in the *Mlh1* or *Apc* gene (29,36,37). These studies show that *Dnmt1* plays an essential role for tumor initiation in these mouse models. Using our REMOTE-control system with the Apc^{Min} model, we found that intestine-specific reduction of *Dnmt1* expression down to 15% resulted in substantial suppression of intestinal polyp formation (Figure 6E). Unlike previous studies using *Dnmt1* hypomorphic mouse models where *Dnmt1* expression is reduced uniformly across all cell types, our system achieves intestinal epithelial cell-specific *Dnmt1* repression. This allows us to attribute the observed strong intestinal antineoplastic effect of *Dnmt1* repression specifically to epithelial cell-autonomous effects. This result not only demonstrates the *in vivo* functionality

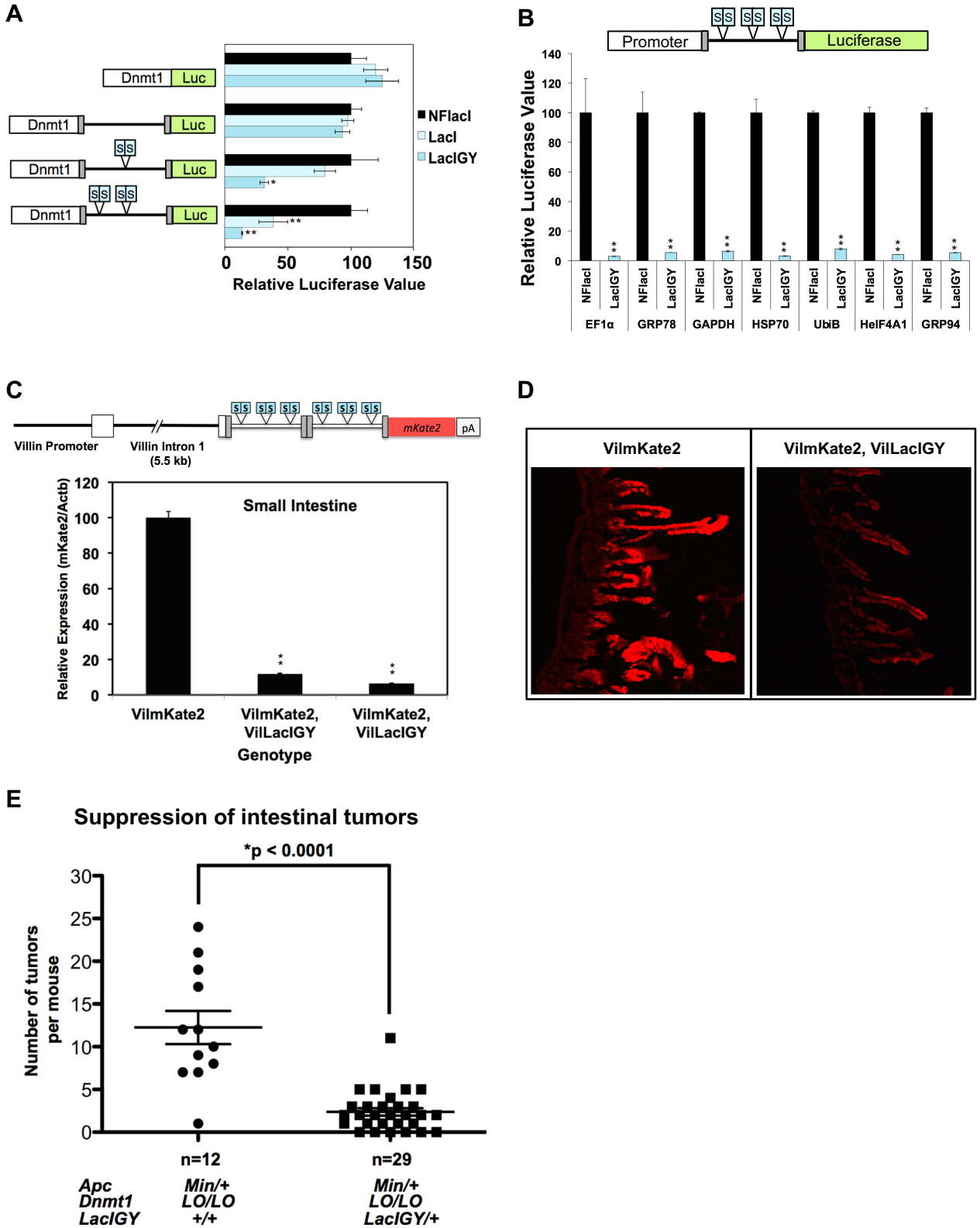

Figure 6. Attenuating transcription elongation by *lac* repressor and applying the repression system to other promoters. (**A**) Attenuation of transcription elongation. The rabbit β-globin intron (black line flanked by gray boxes) was modified with symmetric operators under the control of the *Dnmt1* promoter (one-way-ANOVA). (**B**) Attenuation of transcription elongation of various promoters (*t*-tests). Two symmetric *lac* operators were inserted at three locations within a rabbit β-globin intron. Luciferase data are presented as percent relative to the reference value set to 100%. (**C**) The *VilmKate2* transgenic mouse line. The rabbit β-globin intron was modified with symmetric operators under the control of the *Villin* promoter. *mKate2* expression in the small intestine from mice with or without *lacIGY* (one-way ANOVA). (**D**) Confocal *mKate2* images of small intestine with and without *lacIGY* expression. (**E**) Tumor multiplicity in the Apc^{Min} mice analyzed for tumor multiplicity at six months of age (Mann–Whitney U test). Data are represented as mean ± SEM ($n = 3$). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$

and potency of the system, but also provides new experimental opportunities that the system has to offer for investigating the role of DNA methylation beyond tumorigenesis. For example, since the system allows a reversible control, it is now possible to ask whether *Dnmt1* also plays a role in the maintenance of the established tumors. The mouse model can be maintained upon IPTG treatment to allow tumors to grow under normal *Dnmt1* expression, and IPTG can be withdrawn to repress *Dnmt1* expression after tumors are formed. The induction kinetics of IPTG (2–3 days) and of Doxycycline (6–10 days) are sufficiently responsive for such tumor initiation and maintenance studies (Supplementary Figure S4A) (2,38–40). This question is particularly relevant in light of the increasing interest in the use of epigenetic therapy in human malignancies.

DISCUSSION

Significant effort has been expended to achieve more refined control of gene expression to recapitulate pathological processes or to experimentally intervene in biological pathways with precision. Advances in molecular genetics enable temporo-spatial dissection of gene function by conditionally activating or inactivating target genes in living organisms. Site-specific binary recombination technologies, such as the Cre/*loxP* system, have been widely used to achieve conditional inactivation. The Cre/*loxP* system now allows for inducible activation through conditional excision of a stop element in the target gene (41). However, in these recombination-based approaches, the ability to control gene function is lost once deletion has taken place. Further, the dichotomous nature of the system does not allow assessment of phenotypic effects across a continuous spectrum of gene expression levels.

The REMOTE-control system offers reversible and tunable expression control that can be applied and withdrawn multiple times in a living organism, allowing us to test the reversibility of a phenotype and to investigate gene function at different expression levels. The potency and multi-functionality of the REMOTE-control system makes it an attractive alternative to conventional and conditional knockout approaches.

Control of endogenous gene expression has also been achieved by several fusion transcriptional activators or repressors, such as KRAB-fused or VP64-fused ZNF, TALE or dCas9 proteins (6,42,43). Of these, the dCas9 system offers the greatest flexibility and potential, allowing the targeting of any genomic loci by introducing RNA sequences complementary to the regions of interest. However, the recruitment of powerful eukaryotic repressors, such as the KRAB and its interacting protein KAP1, results in permanent silencing of target genes by inducing promoter DNA methylation and heterochromatin, which can spread over several tens of kilobases, (44,45). In contrast, the REMOTE-control system achieves repression by steric hindrance (46). Indeed, we were able to reverse the repression caused by the *lacIGY* repressor after several months of suppression in mice (see Figure 4D and E). Notably, we were also able to fully restore expression from the targeted allele after over two months of repression in heterozygote ES cells ($Dnmt1^{LGT/+}$) (Supplementary Figure S6B), which maintain global DNA methylation levels comparable to those of wild-type ES cells (see Supplementary Figure S5A and B) (47). This ability to temporarily affect endogenous gene expression should provide greater precision for investigating gene function *in vivo*.

In this study, our enhanced *lac* system was able to overcome the transcriptional strength of all the robust promoters tested, suggesting promising general applicability and reliability. A limitation of the REMOTE-control is the potential challenge associated with the insertion of the effector binding sites without affecting target gene expression. Prokaryotic repressors achieve repression by steric hindrance with the transcription machinery (48). This short-range mode of action imposes constraints on the design of the target promoter configuration, and increases the risk of affecting transcription in the absence of repressor binding while our enhanced *lac* repressor successfully repressed both CMV and *Dnmt1* promoters from operators located outside the critical promoter regions. Our intron-based alternative repression approach substantially mitigates this risk and thus improves the general applicability of the REMOTE-control system. The enhanced *lac* system was also able to tightly repress transcription at operators located several kilobases downstream of the transcription start site (Figure 6C and D). Importantly, this approach may be less susceptible to variation among different genes, tissues, and organisms because the repression is likely achieved through physical hindrance between two components, the Pol II transcription elongation machinery and the lac repressor–operator complexes (Figure 6B–D, Supplementary Figure S7B and C) (46). The large evolutionary distance to the originating species of the regulatory components and the complexity of the operator sequences provides for a high degree of target specificity in the control of gene expression in mammals (49). The availability of regulatory ligands (IPTG for the *lac* repressor and doxycycline for the *tet* activator) provides temporal and dose-dependent expression control. Furthermore, libraries of repressors and activators can be established and applied to any endogenous gene with predictable spatial and/or temporal regulation. For example, existing *tet* transactivator transgenic mouse lines can be readily applied to achieve upregulation of the endogenous *Dnmt1* gene in most mouse tissues, which will be a useful resource because the gene plays important roles in many biological and pathological processes, including cancer.

Gene upregulation by the REMOTE-control system has several advantages over other inducible transgenic approaches. It eliminates the necessity of generating multiple transgenic lines to rule out position effects of the transgene insertion, as the upregulation is achieved at the endogenous locus. In addition, our approach is well-suited for genes with a robust basal expression because it enhances expression from an already strong endogenous promoter, whereas other transgenic approaches induce expression from minimal viral promoters. In this study, we were able to increase the activity of the robust *Dnmt1* promoter up to 6.5-fold (see Figure 5C-E). We were even able to upregulate the *EF1*α promoter, one of the strongest known promoters, 3.5-fold (see Supplementary Figure S7D), which would be difficult to achieve with minimal viral promoters. Finally, our ap-

proach retains elements of natural regulation, including innate *cis*-regulatory elements, which may confer tissue specificity, cell-cycle control, and splicing variants, which would be difficult to achieve with transgenic approaches. On the other hand, our approach might have a limitation in inducing expression from promoters with low transcription potential, such as ones in strong heterochromatic regions as indicated by our results from mouse heart (Figure 5E). It remains to be tested whether such promoters can also be upregulated with higher levels of activator and more binding sites.

The potency and multi-utility of our system provide the capability to control the level, timing, and location of endogenous gene expression and will enable *in vivo* functional analyses at much higher resolutions. With the recent advent of CRISPR/Cas-mediated gene-targeting technology, genetic manipulation of endogenous loci has been readily accomplished (13–16). This technical advance will greatly facilitate the insertion of regulatory sequences for the REMOTE-control system and will enable facile application of the technology.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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