Cre-lox-regulated conditional RNA interference from transgenes

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We have generated two lentiviral vectors for conditional, Cre-lox-regulated, RNA interference. One vector allows for conditional activation, whereas the other permits conditional inactivation of short hairpin RNA (shRNA) expression. The former is based on a strategy in which the mouse U6 promoter has been modified by including a hybrid between a LoxP site and a TATA box. The ability to efficiently control shRNA expression by using these vectors was shown in cell-based experiments by knocking down p53, nucleophosmin and DNA methyltransferase 1. We also demonstrate the usefulness of this approach to achieve conditional, tissue-specific RNA interference in Cre-expressing transgenic mice. Combined with the growing array of Cre expression strategies, these vectors allow spatial and temporal control of shRNA expression *in vivo* and should facilitate functional genetic analysis in mammals.

RNA interference (RNAi) has emerged as a powerful tool to silence gene expression, and has rapidly transformed gene function studies across phyla. RNAi operates through an evolutionarily conserved pathway that is initiated by double-stranded RNA (dsRNA; for review, see refs. 1 and 2). In model eukaryotes such as plants and worms, long dsRNA (e.g., 1,000 bp) introduced into cells is processed by the dsRNA endoribonuclease Dicer into ≈21-nt small-interfering RNAs (siRNAs). siRNAs in turn associate with an RNAi-induced silencing complex and direct the destruction of mRNA complementary to one strand of the siRNA. Although the Dicer pathway is highly conserved, introduction of long dsRNA (>30 bp) into mammalian cells results in the activation of antiviral pathways, leading to nonspecific inhibition of translation and cytotoxic responses (3). The use of synthetic siRNAs to transiently down-modulate target genes, is one way to circumvent the cytotoxic dsRNA-activated pathways in mammals (4).

An important advance in the RNAi field was the discovery that plasmid-based RNAi can substitute for synthetic siRNAs, thus permitting the stable silencing of gene expression (5). In such systems, an RNA polymerase III promoter is used to transcribe a short stretch of inverted DNA sequence, which results in the production of a short hairpin RNA (shRNA) that is processed by Dicer to generate siRNAs. These vectors have been widely used to inhibit gene expression in mammalian cell systems.

More recently, several groups have reported the use of RNA polymerase III-based shRNA expression constructs to generate transgenic RNAi mice (6–8), in some cases recapitulating knockout phenotypes (7, 8). Due to the dominant nature of RNAi, a major limitation of this approach is that germ-line transmission can be obtained only for shRNAs targeting genes whose knock-down is compatible with animal viability and fertility. Moreover, even for cell-based applications, constitutive knock-down of gene expression by RNAi can limit the scope of experiments, especially for genes whose inhibition leads to cell lethality.

To overcome these limitations, and to extend the applications of RNAi in mammalian systems, we have developed a Cre-lox-based approach for the conditional expression of shRNA. Two different strategies were used to generate mouse embryonic fibroblasts (MEFs), embryonic stem (ES) cells and transgenic mice in which the expression of an shRNA is tightly regulated in a Cre-dependent manner. One vector allows for conditional activation of shRNA expression, whereas the other permits conditional inactivation of expression of the hairpin RNA. When combined with a variety of Cre expression strategies, these vectors add a powerful capability in the use of RNAi to control mammalian gene expression.

Materials and Methods

Generation of Plasmids. To generate the plasmid for stable RNAi, *co*nditional (pSico), the lox-CMV-GFP-lox cassette was removed from lentilox 3.7 (pLL3.7; ref. 7) by digesting with *Bfu*AI and *Pci*I, followed by filling-in and religation. The first bifunctional lox site (hereafter termed "TATAlox"), followed by the terminator and by an *Eco*RI, was inserted in the resulting plasmid by PCR-mediated mutagenesis using the following oligos: pSico6Eco, GAATTCAACGCGCGGTGACCCTCGAGG; and pSico6, ASAAAAAACCAAGGCTTATAACTTC-GTATAATTTATACTATACGAAGTTATAATTACTTTA-CAGTTACCC.

To insert the second TATAlox preceded by a *Not*I site, the resulting plasmid was digested with *Eco*RI and *Xho*I and ligated to the following annealed oligos: TATALOX F, AATTCGA-GAGGCGGCCGCATAACTTCGTATAGTATAAATTA-TACGAAGTTATAAGCCTTGTTAACGCGCGGTGACCC; and TATALOX R, TCGAGGGTCACCGCGCGTTAACAA-GGCTTATAACTTCGTATAATTTATACTATACGAA-GTTATGCGGCCGCCTCTCG.

The resulting construct was finally digested with *Eco*RI and *Not*I and ligated to an *Eco*RI-CMV-GFP-*Not*I cassette to generate pSico. A similar strategy was used to generate the various "test" constructs shown in Fig. 6, which is published as supporting information on the PNAS web site. Primer sequence and details are available upon request.

To generate pSico Reverse (pSicoR) the 5′ loxP site present in pLL3.7 was removed by digesting with *Xho*I and *Not*I and replaced with a diagnostic *Bam*HI site by using the following annealed oligos: Lox replace for TCGAGTACTAGGATCCAT-TAGGC and Lox replace rev GGCCGCCTAATGGATCCT-AGTAC.

A new lox site was inserted 18 nt upstream of the proximal sequence element (PSE) in the U6 promoter by PCR-mediated mutagenesis.

Abbreviations: shRNA, short hairpin RNA; siRNA, small interfering RNA; RNAi, RNA interference; ES, embryonic stem; Npm, nucleophosmin; Dnmt1, DNA methyltransferase 1; MEF, mouse embryonic fibroblasts; dsRNA, double-stranded RNA; pSico, plasmid for stable RNAi, conditional; pSicoR, pSico reverse; PSE, proximal sequence element; DSE, distal sequence element; TATAlox, a bifunctional lox site; Ad, adenovirus; Ad-Cre, Cre-expressing recombinant Ad.

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Oligos coding for the various shRNAs were annealed and cloned into *Hpa*I–*Xho*I-digested pLL3.7, pSico, and pSicoR. Oligo design was as described (7). The following target regions were chosen: Nucleophosmin (Npm), GGCTGACAAAGACTATCAC; Luciferase, GAGCTGTTTCTGAGGAGCC; DNA methyltransferase 1 (Dnmt1), GAGTGTGTGAGGGAGAAA; and P53, GTACTCTCCTCCCCTCAAT.

The CD8 oligo sequence was the same described in ref. 7. All constructs were verified by DNA sequencing. To amplify recombined and unrecombined vector the following oligos were used: Loopout F, CCCGGTTAATTTGCATATAATATTTC; and Loopout R, CATGATACAAAGGCATTAAAGCAG.

Virus Generation and Infection. Lentiviruses were generated essentially as described (7). Briefly, 5 μg of lentiviral vector and 2.5 μg of each packaging vector were cotransfected in 293T cells by using the FuGENE 6 reagent (Roche Diagnostics). Supernatants were collected 36–48 h after transfection, filtered through a 0.4-μm filter, and used directly to infect MEFs. Two rounds of infection 8 h apart were usually sufficient to infect >90% of cells. GFP-positive cells were sorted 3–4 days after infection. For ES cell infection, the viral supernatant was centrifuged at 25,000 rpm in a Beckman SW41t rotor for 1.5 h, the viral pellet was resuspended in 200 μl of ES cell medium, and was incubated 6 h at 37°C with 10,000–20,000 cells. After infection, ES cells were plated in 10-cm dishes with feeders and GFP-positive colonies were isolated 4–5 days later. On average, 10–30% of ES colonies were GFP-positive.

Recombinant adenoviral stocks were purchased from the Gene Transfer Vector Core facility of University of Iowa College of Medicine (Iowa City, IA). Infections were performed by using 100 plaque-forming units of virus per cell.

ES Cell Manipulation, Generation of Chimeras, and Tetraploid Complementation. V6.5 ES cells were cultivated on irradiated MEFs in DMEM containing 15% FCS, leukemia-inhibiting factor, penicillin/streptomycin, L-glutamine, and nonessential amino acids. MEFs were cultivated in DMEM and 10% FCS supplemented with L-glutamine and penicillin/streptomycin. The derivative of V6.5 containing a doxycycline-inducible Cre transgene in the collagen locus will be described elsewhere (C. Beard and R.J., unpublished data).

B6D2F2 diploid blastocysts and B6D2F2 tetraploid blastocysts were generated and injected with ES cells as described (9). Tetraploid blastocyst-derived animals were delivered by cesarean section on postnatal day 19.5 and fostered to lactating BALB/c mothers. Alternatively, embryonic day 14.5 embryos were surgically removed to generate MEFs following standard procedure. Msx2-Cre mice (10) were received from G. Martin (University of California, San Francisco) and Lck-Cre mice (11) were obtained from The Jackson Laboratory.

Southern Blot and Methylation Analyses. DNA was isolated from the indicated ES cell lines. To assess the levels of DNA methylation, genomic DNA was digested with *Hpa*II and was hybridized to pMR150 as a probe for the minor satellite repeats (12). For the methylation status of imprinted loci, a bisulfite conversion assay was performed by using the CpGenome DNA modification kit (Chemicon), using PCR primers and conditions already described (13). PCR products were gel-purified, digested with *Bst*UI, and resolved on a 2% agarose gel.

Northern Blots. For the small RNA Northern blotting, 15 μg of total RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions, and was resolved on a 15% denaturing polyacrylamide gel, transferred to a nylon membrane, and was cross linked by using the autocrosslink function of a Stratalinker. The membrane was hybridized overnight to a ^{32}P 5′-labeled DNA probe corresponding to the 19-nt sense strand of the p53 shRNA (GTACTCTCCTCCCCTCAAT). Hybridization and washes were performed at 42°C.

For detection of the p53 mRNA, 15 μg of total RNA was resolved on an agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized to a probe corresponding to the entire p53 coding sequence.

Antibodies, Chemicals, and Flow Cytometry. Anti-α-tubulin antibody was from Sigma, the p53 antibody was a kind gift by K. Helin (European Institute of Oncology, Milan), and the anti-Npm was a gift from P. G. Pelicci (European Institute of Oncology, Milan) and E. Colombo (European Institute of Oncology, Milan). All mouse monoclonal antibodies were used. Doxorubicin and doxycycline were obtained from Sigma.

To assess expression of CD4 and CD8 in mice, single-cell suspensions of splenocytes were blocked with anti-CD16/CD32 for 10 min on ice. After blocking, the cells were incubated with phycoerythrin-conjugated anti-CD8, allophycocyanin-conjugated anti-CD4, and PerCPCy5.5-conjugated anti-CD3 for 20 min at 4°C (BD Pharmingen, San Diego). Acquisition of samples was performed on a FACScan flow cytometer, and the data were analyzed with CELLQUEST software (BD Immunocytometry Systems, San Jose, CA). Plots were gated on CD3$^+$ cells

For cell-cycle analysis, 10^6 cells were fixed in 70% ethanol, washed in PBS, and resuspended in 20 μg/ml propidium iodide (Sigma) and 200 μg/ml RNAseA in PBS.

Luciferase Assay. For reporter assay, 293T cells were cotransfected in 12-well plates by using FuGENE 6 with the appropriate shRNA vectors and pGL3control and pRLSV40. The total amount of transfected DNA was 500 ng per well. Firefly and Renilla luciferase activity were measured 36 h after transfection by using the dual reporter kit (Promega) according to the manufacturer's instruction. All experiments were performed in triplicate.

Results

Generation of pSico and pSicoR. The U6 promoter has been widely used to drive the expression of shRNAs and a U6-based lentiviral vector for the generation of transgenic mice has been recently described (7). To control shRNA expression in a Cre-dependent manner, we decided to modify the mouse U6 promoter by inserting a Lox-STOP-Lox cassette. Similar to other RNA polymerase III promoters, the U6 promoter is extremely compact, consisting of a tightly spaced TATA box, a PSE, and a distal sequence element (DSE; Fig. 1*A*). Mutagenesis experiments have demonstrated that while the DSE is partially dispensable for transcriptional activity, the PSE and the TATA box are absolutely required. Moreover, the spacing between the PSE and the TATA box (17 nt) and between the TATA box and the transcription start site (25 nt) is critical, because even small changes have been shown to severely impair promoter activity (14). A consequence is that to effectively suppress the activity of the U6 promoter, the Lox-STOP-Lox element must be positioned either between the PSE and the TATA box or between the TATA box and the transcription start site. In addition, to reconstitute a functional promoter, after Cre expression, the normal spacing between PSE, TATA box, and transcription start site must be restored. The latter consideration precludes the utilization of a classic lox-STOP-lox cassette because, after Cre-mediated recombination, the residual loxP site (34 nt) would necessarily increase the PSE-TATA or the TATA-start-site spacing (See Fig 6).

To overcome these limitations, we generated a bifunctional lox site (TATAlox), that, in addition to retaining the ability to undergo Cre-mediated recombination, contains a functional TATA box in its spacer region (Fig. 1 *B–D*).

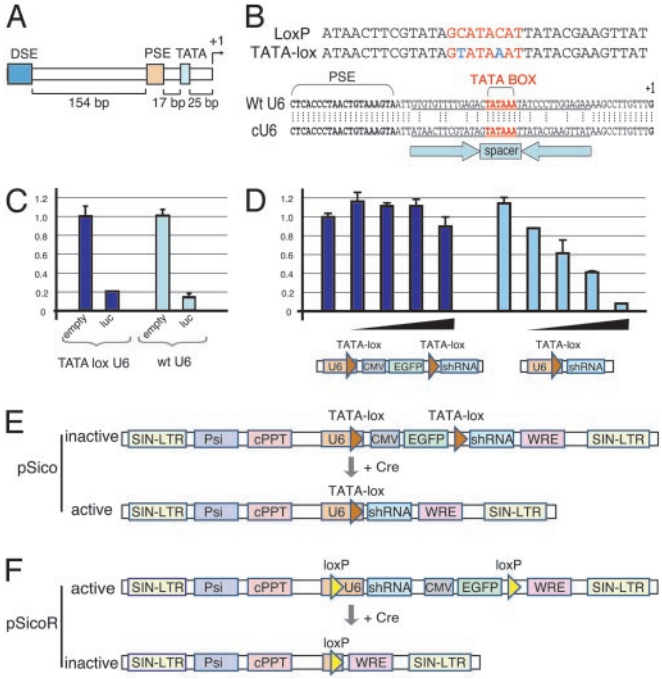

Fig. 1. Generation of pSico and pSicoR. (*A*) Schematic representation of the mouse U6 promoter. The spacing between the DSE, the PSE, the TATA box, and the transcription start site (+1) is indicated. (*B*) Comparison between the sequence of a loxP site and a TATAlox site (*Upper*). Comparison between the sequence of the wild-type mouse U6 promoter and the sequence of the U6 promoter with a TATAlox site replacing the TATA box (*Lower*). (*C*) The TATAlox can replace the TATA box in the U6 promoter. Equal amounts of the wild-type U6 promoter and of the TATAlox U6 promoter (empty or driving the expression of shRNA against the firefly luciferase gene) were transfected in 293T cells together with reporter plasmids expressing firefly luciferase and renilla luciferase. Thirty-six hours later, cells were lysed and the ratio between firefly and renilla luciferase activity was measured. (*D*) A TATAlox-STOP-TATAlox cassette in the U6 promoter efficiently suppresses shRNA expression. Increasing amounts (0–200 ng) of plasmids containing the indicated version of the U6 promoter were transfected in 293T cells together with reporter plasmids, and luciferase activity was measured as in *C*. (*E*) Schematic representation of pSico before and after Cre-mediated recombination. (*F*) Schematic representation of pSicoR before and after Cre-mediated recombination. SIN-LTR, self-inactivating long terminal repeats; Psi, required for viral RNA packaging; cPPT, central polypurine tract; EGFP: enhanced GFP; WRE, woodchuck regulatory element.

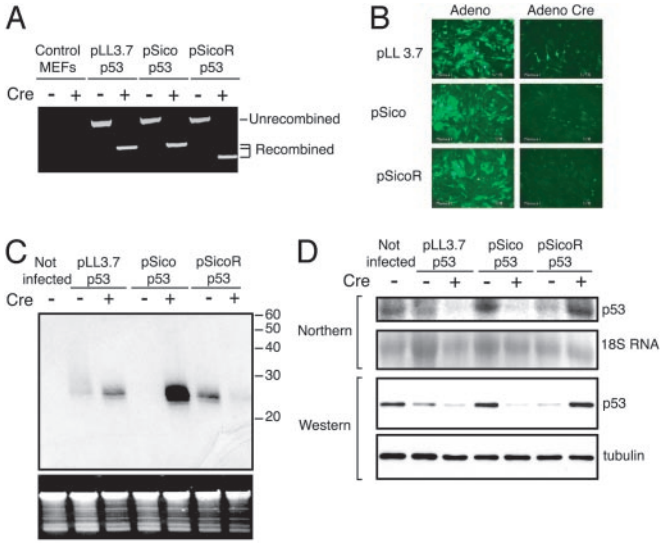

Fig. 2. Cre-regulated knockdown of p53. (*A*) p53 $^{R270H/-}$ MEFs infected with the indicated lentiviruses were sorted for GFP positivity and infected with Ad or Ad-Cre. Four days after infection, genomic DNA was extracted, and a PCR was performed to amplify the recombined and unrecombined viral DNA. (*B*) The same cells were analyzed by epifluorescence microscopy to detect GFP. Similar cell density and identical exposure time was used for all images. (*C*) Fifteen micrograms of total RNA extracted from the above indicated MEFs was separated on a 15% denaturing polyacrylamide gel, transferred on a nitrocellulose filter, and hybridized to a radi-labeled 19mer corresponding to the sense strand of the p53 shRNA. Equal RNA loading was assessed by ethidium bromide staining of the upper part of the gel (*Lower*). (*D*) Northern (*Upper*) and Western blotting (*Lower*) showing p53 knock-down in the above indicated cells.

As shown in Fig. 1, when the TATAlox replaces the TATA box site in the U6 promotor, the spacing between PSE, TATA, and transcriptional start site is not altered (Fig. 1*B*), and the resulting promoter retains transcriptional activity (Fig. 1*C*).

To create a conditional U6 promoter, a cytomegalovirus (CMV)-enhanced GFP stop/reporter cassette was inserted between two TATAlox sites so that after Cre-mediated recombination the cassette would be excised, generating a functional U6 promoter with a TATAlox in place of the TATA box (Fig. 1*D*). A T_6 sequence was positioned immediately upstream of the CMV promoter to serve as a termination signal for RNA polymerase III. The terminator combined with the inserted CMV-GFP cassette completely suppressed the activity of the U6 promoter (Figs. 1*D* and 2 *C* and *D*). To facilitate the generation of conditional knock-down mice and cell lines, the conditional U6 cassette was inserted into a self-inactivating lentiviral vector derived from pLL3.7 (7). The resulting plasmid was named pSico (Fig. 1*E*).

To allow for conditional inactivation of shRNA expression, we generated a second vector named pSicoR (Fig. 1*F*). In pSicoR, the CMV-GFP reporter cassette is placed downstream of the U6 promoter and does not affect its activity. Two loxP sites in the same orientation are present in this vector; the first positioned immediately upstream of the PSE in the U6 promoter, and the second immediately downstream of the GFP-coding sequence. In contrast to cells infected with pSico, cells infected with pSicoR are expected to constitutively transcribe the desired shRNA until a Cre-mediated recombination event leads to the excision of the CMV-GFP cassette and an essential part of the U6 promoter. Importantly, in both pSico and pSicoR, the CMV-GFP cassette marks infected cells and loss of GFP expression indicates successful Cre-mediated recombination.

Cre-Regulated RNAi in Cells. The ability of pSico and pSicoR vectors to conditional silence endogenous genes was demonstrated by insertion of a hairpin designed to inhibit expression of the mouse tumor suppressor gene p53. As a control, the same sequence was cloned into the constitutive shRNA vector pLL3.7. In pLL3.7, the CMV-GFP cassette is located downstream of the U6 promoter and is flanked by loxP sites such that Cre-mediated recombination is expected to result in loss of GFP expression without affecting shRNA expression (7). These three constructs were then used to generate lentiviruses and infect MEFs. To simplify the detection of p53, MEFs expressing high basal levels of a transcriptionally inactive point mutant (R270H) p53 allele (K. Olive and T.J., unpublished work) were used in these experiments. High-efficiency transduction by all of these vectors was achieved as indicated by uniform GFP expression in infected cells (Fig. 2*B* and data not shown). As shown in Fig. 2, after superinfection with a Cre-expressing recombinant adenovirus (Ad-Cre), near complete recombination with concomitant loss of GFP fluorescence was observed for all vectors. One week after Cre expression, high levels of the p53-siRNA were detected in cells infected with pSico-p53 (Fig. 2*C*), whereas no p53-siRNA

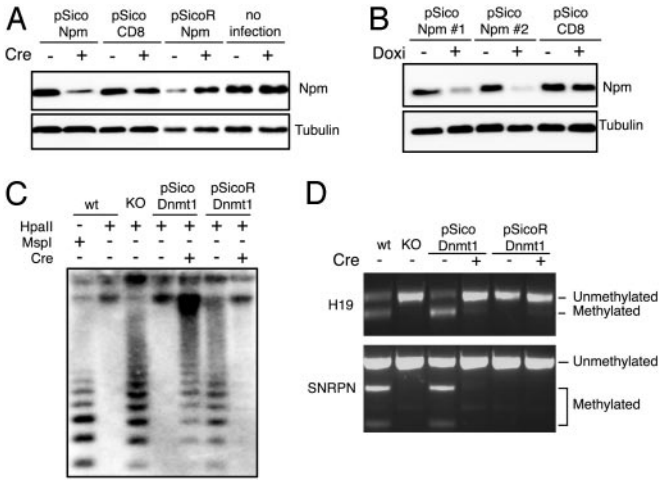

Fig. 3. Cre-regulated knockdown of Npm and Dnmt1. (*A*) Cre-regulated knock-down of Npm. MEFs were infected with the indicated lentiviruses, and GFP-positive cells were sorted and were superinfected with empty Ad or Ad-Cre. One week later, whole-cell lysates were separated by SDS/PAGE, and were subjected to Western blotting against Npm and tubulin. (*B*) ES cells carrying a doxycycline-inducible Cre (C. Beard and R.J., unpublished data) were infected with the indicated lentiviruses. GFP-positive clones were isolated, passaged two times, and were either left untreated or were incubated with 2 μg/ml doxycycline for 1 week. Immunoblot analysis was performed as in *A*. (*C*) Cre-regulated knock-down of Dnmt1 affects cytosine methylation. Methylation analysis of minor satellite DNA. ES cells carrying a doxycycline-inducible Cre transgene were infected with the indicated lentiviruses. Single GFP-positive clones were isolated, expanded, and passaged five times before being either mock-treated or incubated with 2 μg/ml doxycycline. After five more passages, the genomic DNA was extracted and digested with the indicated enzymes and subjected to Southern blot analysis. (*D*) As in *C*, but the genomic DNA was treated with sodium bisulfite, subjected to PCR to amplify the indicated imprinted regions, and digested with *Bst*UI.

was observed in the same cells in the absence of Cre expression, confirming the complete suppression of U6 promoter activity by the TATAlox-STOP-TATAlox cassette. The length of the processed RNA (21–24 nt) was identical in cells infected with pLL3.7-p53, pSico-p53 (after Ad-Cre infection), or pSicoR-p53 (before Ad-Cre infection), indicating that the presence of the TATAlox in pSico did not qualitatively affect siRNA production. Finally, infection with Ad-Cre led to almost complete disappearance of p53-siRNA in pSicoR-p53-infected cells (Fig. 2*C*).

Consistent with functional p53-siRNA expression by these vectors, Cre-mediated recombination resulted in a dramatic reduction of both p53 mRNA and protein levels in pSico-p53-infected cells (Fig. 2*D*). Conversely, pSicoR-p53 generated a p53 knock-down that was reversed upon Ad-Cre infection (Fig. 2*D*). We noticed an unexpected increase in p53-siRNA and p53 knock-down after Cre expression in cells infected with pLL3.7-p53 (Fig. 2 *C* and *D*, lanes 2 and 3). This increase could reflect promoter interference because the CMV and the U6 promoters are in close proximity in pLL3.7 before Cre-mediated recombination.

As additional proof of concept, we cloned short hairpins directed against the nucleolar protein Npm and the DNA methyl transfrase Dnmt1 into pSico and pSicoR. Npm is a putative tumor-suppressor gene involved in a number of chromosomal translocations associated with human leukemias and lymphomas, and has been shown to physically and functionally interact with the tumor suppressors p19ARF and p53 (15, 16). Specific, Cre-dependent knock-down of Npm was observed in both MEFs and ES cell clones infected with pSico-Npm (Fig. 3 *A* and *B*). The opposite effect, Cre-dependent reexpression of Npm, was observed in pSicoR-Npm-infected MEFs (Fig. 3*A*, and Fig. 7, which is published as supporting information on the PNAS web site).

The characterization of ES cells mutant for Dnmt1 has been reported (17), and demonstrated that Dnmt1 is required for genome-wide maintenance of cytosine methylation. Dnmt1-deficient ES cells are viable and proliferate normally, despite substantial loss of cytosine methylation; however, they die upon differentiation. Whereas reexpression of the Dnmt1 cDNA in these cells leads to methylation of bulk genomic DNA and nonimprinted genes, the methylation pattern of imprinted loci cannot be restored without germ-line passage (18, 19). We tested whether we could recapitulate the phenotype observed in Dnmt1-deficient ES cells by using pSico-Dnmt1 and pSicoR-Dnmt1. As shown in Fig. 3, pSico-Dnmt1-infected ES cells underwent significant loss of CpG methylation of minor satellites (Fig. 3*C*) and of two imprinted genes tested (Fig. 3*D*) upon Cre induction. Importantly, the reacquisition of DNA methylation at minor satellite sequences, but not at imprinted loci in pSicoR-Dnmt1 after Cre-mediated recombination, confirms previous results obtained with reexpression of Dnmt1 (19). These results further illustrate the potential for application of the pSicoR vector *in vitro* and *in vivo* to perform "rescue" experiments.

Conditional RNAi in Mice. One motivation for incorporating a conditional U6 cassette into a lentiviral vector was to rapidly generate conditional knock-down mice. To demonstrate this application directly, ES cells were infected with pSico-CD8 (Fig. 4*A*), which was designed to inhibit expression of the T lymphocyte cell surface marker CD8 (7). Three pSico-CD8 ES clones were used to generate chimeric mice, and transmission of the pSico-CD8 transgene to the progeny was observed for two of them. All transgenic mice were easily identified by macroscopic GFP visualization (Fig. 4*B*), although we observed some variability in the extent and distribution of GFP expression among littermates. Importantly, all transgenic mice produced normal amounts of $CD4^+$ and $CD8^+$ lymphocytes and were apparently normal and fertile, indicating that the presence of the nonexpressing pSico-CD8 transgene before Cre activation did not affect CD8 expression and was compatible with normal mouse development.

To achieve either global or tissue-specific activation of the CD8 shRNA, pSico-CD8 chimeras were crossed to Msx2-Cre or Lck-Cre transgenic mice that express Cre in the oocyte (10, 20), or under the control of a T cell-specific promoter (11), respectively. Fluorescence-activated cell sorter analysis demonstrated that pSico-CD8;Lck-Cre and pSico-CD8;Msx2-Cre mice had a specific reduction in splenic $CD8^+$, but not $CD4^+$ T lymphocytes as compared with controls (Fig. 4*C*). As predicted, the pSico-CD8;Msx2-Cre progeny showed complete recombination of the pSicoCD8 transgene and lacked detectable GFP expression, although in the pSico-CD8;Lck-Cre mice recombination was detected in the thymus but not in other tissues (Fig. 4*D* and data not shown). Transgenic mice derived from two different ES clones gave similar results.

Tetraploid blastocyst complementation represents a faster alternative to diploid blastocyst injection because it allows the generation of entirely ES-derived mice without passage through chimeras (9, 21). In principle, this technology applied to pSico-infected ES cells would allow the generation of conditional knock-down mice in ≈5–6 weeks (1 week for cloning the shRNA, 1–2 weeks for ES cell infection and clone selection, and ≈2 weeks for tetraploid blastocyst injection and gestation). To test this protocol directly, ES cells were infected with pSico-p53 and two different clones, pSico-p53#1 and pSico-p53#3, were injected into tetraploid blastocysts. As a rapid way to assess the inducibility of the p53 shRNA in ES cell-derived animals, midgestation embryos were recovered from two recipient females. Two apparently normal, GFP-positive embryos were recovered; one

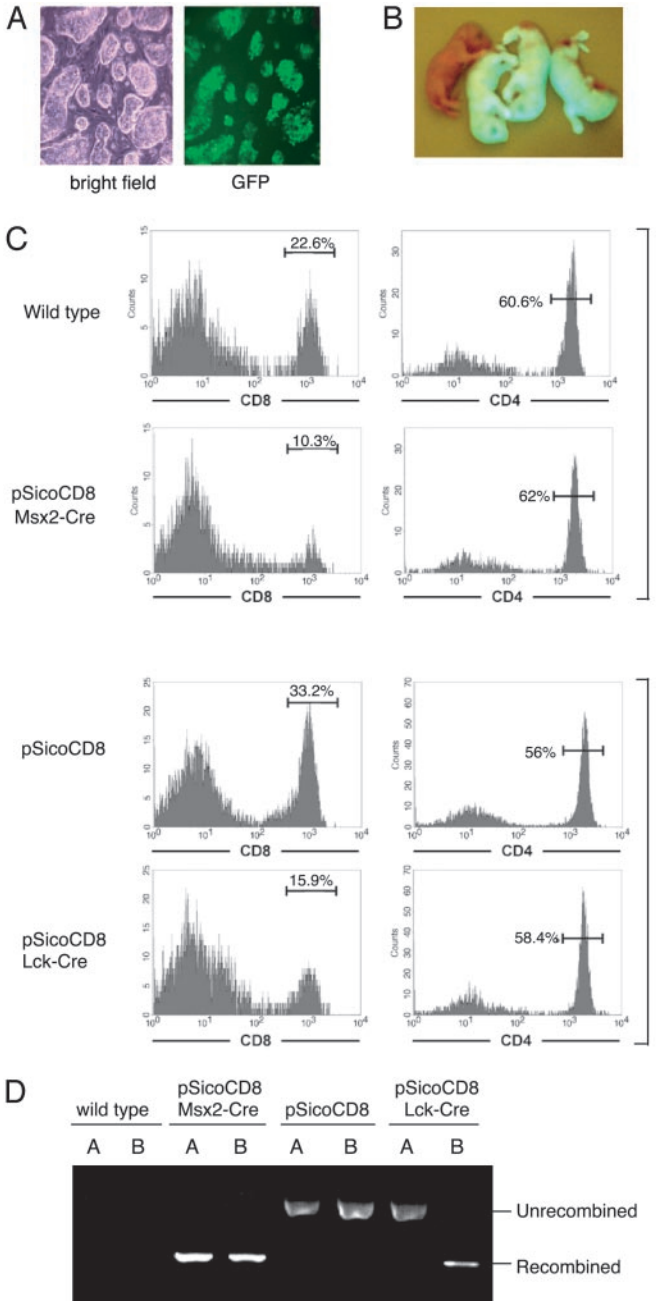

Fig. 4. Conditional knockdown of CD8 in transgenic mice. (*A*) ES cells infected with pSico-CD8 visualized with an inverted fluorescence microscope. (*B*) A litter of newborns derived from a cross between a pSico-CD8 chimera and an Lck-Cre female. Three pups present bright GFP fluorescence, indicating germ-line transmission of the pSico-CD8 transgene. (*C*) Knock-down of CD8 in the spleen of Msx2-Cre × pSico-CD8 and Lck-Cre × pSico-CD8 mice. Chimeras from pSico-CD8-infected ES cells were crossed to Msx2-Cre or Lck-Cre animals. The resulting mice were genotyped for the presence of Cre and pSico. Splenocytes from 1- to 3-week old mice with the indicated genotypes were harvested, stained for CD3, CD4, and CD8 expression, and analyzed by flow cytometry. Only $CD3^+$ cells were plotted. One representative example of littermates for each cross is shown.(*D*) PCR detection of Cre-mediated recombination of pSico-CD8 in genomic DNA extracted from the tail (*A*) or the thymus (*B*) of mice with the indicated genotypes.

each from ES clone pSico-p53 #1 and pSico-p53 #3 (Fig. 5*A* and data not shown). MEFs generated from these embryos were passaged once and infected with Ad or Ad-Cre. As expected, Cre expression induced significant recombination and loss of GFP

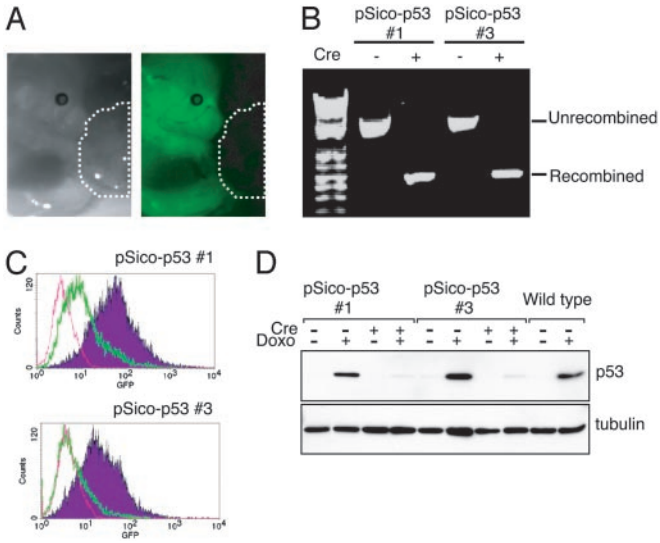

Fig. 5. Generation of conditional knockdown embryos by tetraploid complementation. (*A*) A postnatal day 14.5 embryo derived by tetraploid complementation using the pSico-p53 #1 ES clone. The area enclosed by the dashed line corresponds to the non-ES cell-derived placenta. (*B*) PCR detection of recombination in MEFs derived from the indicated embryos. Genomic DNA was extracted 4 days after Ad or Ad-Cre infection and subjected to PCR. (*C*) Histogram overlays showing loss of GFP expression in MEFs derived from pSico-p53#1 (*Upper*) and pSico-p53#3 (*Lower*) embryos 4 days after Ad-Cre (green plot) or Ad empty (purple filled plot) infection. Control, GFP-negative MEFs (red plot) are included as reference. (*D*) MEFs derved from the indicated tetraploid complementation pSico-p53 embryos, or from wild-type embryos, were treated with doxorubicin for 18 h and subjected to Western blot against p53 and β-tubulin.

expression (Fig. 5 *B* and *C*). Importantly, in Ad-Cre-infected cells, p53 induction and cell-cycle arrest after doxorubicin treatment were significantly inhibited compared with Ad-infected control cells (Fig. 5*D*, and Fig. 8, which is published as supporting information on the PNAS web site).

Discussion

Here, we describe two lentivirus-based vectors for conditional, Cre-lox-regulated RNAi in cells and mice; one for Cre-dependent activation (pSico) and one for Cre-dependent termination (pSicoR) of shRNA expression. These vectors were used to demonstrate conditional and reversible knock-down of p53, Npm, and Dnmt1 in ES cells and MEFs. As a proof of principle, pSico was used to generate conditional and tissue-specific knock-down mice.

Since the development of gene targeting technologies in ES cells (22), the gold standard for the analysis of gene function in mammals has been the creation of knock-out mice. Improvements to this technology have allowed refined analysis of gene function at specific developmental stages or in specific tissues, based on conditional knock-out strategies by means of Cre-lox-regulated recombination (23). Despite significant improvements over the last decade, however, the creation of loss-of-function alleles in the mouse remains time consuming and costly. The recent demonstration that constitutive expression of shRNAs driven by RNA polymerase III promoters can be used to functionally silence gene expression in transgenic mice suggests that RNAi-based technologies might represent a convenient alternative to gene targeting through homologous recombination (6–8).

A major limitation of current approaches for transgenic RNAi is that they do not allow regulated expression of shRNA, but

instead cause constitutive gene silencing in all tissues. The two lentiviral vectors described here overcome this limitation.

The compact nature of RNA polymerase III promoters (14) prevents the use of a conventional Lox-STOP-lox strategy to achieve Cre-inducible shRNA expression. Other investigators have recently addressed this problem by placing the lox-STOP-lox cassette in the loop region of the shRNA (24, 25). However, by using this approach, after Cre-mediated recombination, the residual loxP site is transcribed within the shRNA, resulting in the synthesis of a longer dsRNA that is significantly less efficiently processed (24) and could be more prone to elicit nonspecific, off-target effects or an IFN response (3). By using a mutant lox site containing a functional TATA box in its spacer sequence, we were able to obtain Cre-regulated transcription and efficient processing of a normal-length shRNA.

We further extend the potential applications of RNAi-based technologies by describing a lentiviral vector (pSicoR) in which constitutive shRNA expression can be terminated by a Cre-mediated recombination event. As we demonstrate for Dnmt1, this vector can be used to determine the functional consequences of gene reactivation and will facilitate rescue experiments *in vivo*. In addition, by mimicking the action of small-molecule drugs designed to activate the proteins or pathways controlled by human disease genes (e.g., tumor suppressor gene), this strategy could be applied to identify promising novel targets for drug development.

Because preparation of conditional RNAi constructs requires merely cloning of short synthetic DNA sequences, a large number of conditional knock-down strains can be generated in parallel by a single investigator. This approach is thus ideally suited for large-scale projects aimed at the characterization of genetic pathways or at the validation of candidate target genes identified through gene profiling screenings. For example, gene expression profiling by using mouse cancer models typically yields numerous genes that distinguish tumor from normal tissue. By using conventional or conditional knockout strategies, it is practical to examine only a small fraction of these genes for functional relevance to tumorigenesis. In contrast, shRNA-conditional systems such as pSico can greatly reduce the time, cost, and effort required to perform experiments of this magnitude.

We note that although in this work pSico and pSicoR were used to control the expression of "artificial" shRNAs, they might also be used to achieve spatially and temporally regulated expression of naturally occurring microRNAs, an approach that could help unravel the biological functions of this abundant class of small RNAs (26).

In summary, the lentiviral vectors reported here represent a significant improvement over constitutive shRNA expression systems and expand the number of potential applications of RNAi-based technologies.

Note. While this manuscript was in preparation, a similar strategy for lentivirus-mediated, Cre-dependent shRNA expression in cells was reported (27). Although our vector and the vector described by Tiscornia *et al.* (27) are both based on a STOP cassette flanked by TATA box-containing lox sites, the presence of the GFP reporter in pSico offers the advantage of marking infected cells and permits the visual detection of successful recombination. Importantly, we show that this strategy can be used to achieve tissue-specific, conditional RNAi in transgenic mice. The demonstration that pSico undergoes efficient tissue-specific recombination both *in vitro* and *in vivo* is of particular relevance because a lox site containing a double mutation in the spacer region identical to the one present in the TATAlox had been previously shown to undergo less efficient recombination compared with a wild-type LoxP site (28).

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